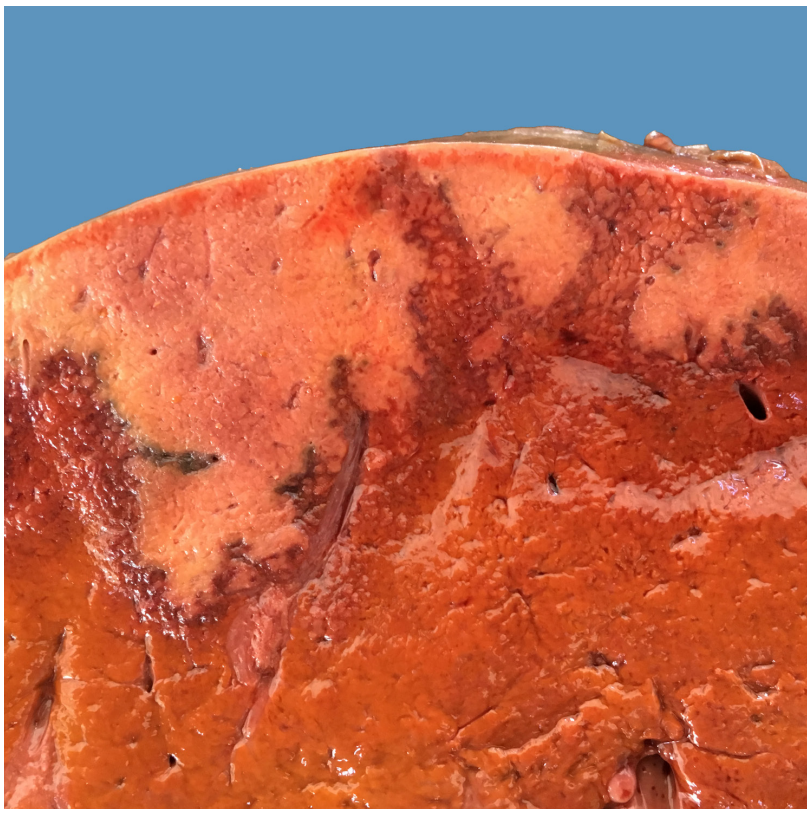
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Cover illustration: At the cut surface of the liver of an ox dead from bacillary hemoglobinuria there is a sizeable pale area of coagulative necrosis with an irregular contour surrounded by a red rim of hemorrhage and inflammation (Santos et al., p. 441).

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Review Article
Livestock Diseases
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Clostridial diseases diagnosed in cattle from the South of Rio Grande do Sul, Brazil. A forty-year survey (1978-2018) and a brief review of the literature[1]

Bianca L. Santos[2], Silvia R.L. Ladeira[2], Franklin Riet-Correa[3], Mauro P. Soares[2], Clairton Marcolongo-Pereira[4], Eliza Simone V. Sallis[5], Margarida B. Raffi[5] and Ana Lucia Schild[2]*

ABSTRACT.- Santos B.L., Ladeira S.R.L., Riet-Correa F., Soares M.P., Marcolongo-Pereira C., Sallis E.S.V., Raffi M.B. & Schild A.L. 2019. **Clostridial diseases diagnosed in cattle from the South of Rio Grande do Sul, Brazil. A forty-year survey (1978-2018) and a brief review of the literature**. *Pesquisa Veterinária Brasileira 39(7):435-446*. Laboratório Regional de Diagnóstico, Universidade Federal de Pelotas, Campus Capão do Leão, Pelotas, RS 96160-000, Brazil. E-mail: alschild@terra.com.br

Clostridial diseases are important causes of livestock losses in the southern Rio Grande do Sul. Since 1978 annual surveys conducted at the "Laboratório Regional de Diagnóstico" of the "Universidade Federal de Pelotas" (LRD-UFPel) have shown that clostridial diseases represent 10.40% of the bacterial diseases diagnosed in cattle and 1.65% of all diseases diagnosis in cattle over a 40-year period. The purpose of this study is to review the clinical, epidemiological and pathological aspects of the clostridial diseases diagnosed in cattle from January 1978 to December 2018 at the LRD-UFPel in the hopes that it will constitute a useful guide for field veterinary practitioners and interested farmers. We assessed and review the necropsy protocols of 6,736 cattle; these necropsies were performed either by LRD-UFPel faculty or by field veterinary practitioners; 111 outbreaks (1.65%) were diagnosed as clostridial disease, distributed as follows: 35 outbreaks of tetanus, 34 of blackleg, 23 of bacillary hemoglobinuria, 11 of malignant edema (gas gangrene), and eight of botulism. Approximately 904, from a total of 42,480 cattle at risk, died in these outbreaks.

INDEX TERMS: Clostridial diseases, diagnosis, cattle, Rio Grande do Sul, Brazil, blackleg, botulism, malignant edema, gas gangrene, bacillary hemoglobinuria, tetanus, *Clostridium* spp.

RESUMO.- [**Clostridioses diagnosticadas em bovinos no sul do Rio Grande do Sul. Uma pesquisa de quarenta anos (1978-2018) e breve revisão da literatura.**] Clostridioses são doenças produzidas por alguma das espécies do gênero *Clostridium* e são importantes causas de perdas pecuárias no sul do Rio Grande do Sul. Pesquisas anuais realizadas no Laboratório Regional de Diagnóstico da Faculdade de Veterinária da Universidade Federal de Pelotas (LRD-UFPel) desde 1978 demonstraram que as clostridioses representaram 11,1% das doenças bacterianas diagnosticadas em bovinos e 1,65% de todos os diagnósticos de doenças em bovinos ao longo de 40 anos. O objetivo deste estudo é revisar os aspectos clínicos, epidemiológicos e patológicos das clostridioses diagnosticadas de janeiro de 1978 a dezembro de 2018, pelo LRD/UFPel com a intenção de que esse trabalho possa servir de guia útil para os veterinários de campo e fazendeiros interessados. Foram avaliados e revisados os protocolos de necropsia de 6.736 bovinos; essas necropsias foram realizadas pelo pessoal do LRD/UFPel ou por veterinários de campo. Cento e quatro (1,16%) casos foram diagnosticados como clostridioses, distribuídos da seguinte forma: 35 surtos de tétano, 34 de cartbúnculo sintomático, 23 de hemoglobinúria

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bacilar, 11 de edema maligno (gangrena gasosa) e oito de botulismo. Aproximadamente 904, de um total de 42.480 bovinos sob-risco, morreram nesses surtos.

TERMOS DE INDEXAÇÃO: Clostridiose, diagnóstico, bovinos, Rio Grande do Sul, Brasil, carbúnculo sintomático, botulismo, edema maligno, gangrene gasosa, hemoglobinúria bacilar, tétano, *Clostridium* spp.

INTRODUCTION

Clostridial diseases are bacterial diseases caused by one or more of the several species of *Clostridium* and their potent toxins. Members of the genus *Clostridium* are gram-positive anaerobic rods that form heat-resistant endospores (Rood 2016).

Although the pathogenesis of clostridial diseases always involves the action of specific toxins produced by the bacteria (with only one exception) they are truly infectious diseases. The infectious agent needs to establish it in the host and overcome its immune defenses so it can grow, multiply and elaborate the toxins (Rood 2016). The exception is botulism, which is usually a true toxemia, being caused by the ingestion of botulinum neurotoxin (BoNT) preformed in food (Guizelini et al. 2019).

Clostridial diseases can be divided into three major types: 1) neurotoxic diseases (Böhmel & Gessler 2010), 2) histotoxic diseases (Songer 2010a) and 3) enteric diseases (Songer 2010b). In cattle, the neurotoxic group includes botulism, due to the ingestion of BoNTs; and tetanus, caused by the infection of *Clostridium tetani* and related neurotoxins (TeNT). The histotoxic group includes diseases such as blackleg (*Clostridium chauvoei*), malignant edema (*Clostridium novyi* type A, *Clostridium perfringens* type A, *Clostridium sordelli, Clostridium septicum*), and bacillary hemoglobinuria (*Clostridium haemolyticum*). The group of enteric diseases includes several types of necrotizing and hemorrhagic enteritis (*Clostridium difficile* in calves, *C. perfringens* type C, *C. perfringens* type E) and enterotoxemia (*C. perfringens* type D) which occurs in small ruminants and possibly in cattle (Uzal et al. 2002, Lobato et al. 2006, Filho et al. 2009, Mete et al. 2013).

Clostridial diseases usually occur with high rates of lethality causing significant economic losses in cattle in the region of influence of the "Laboratório Regional de Diagnóstico" of the "Universidade Federal de Pelotas" (LRD-UFPel), which is located in the South of the state of Rio Grande do Sul (latitude 31º45'48 "South, longitude 52º29'02" West, altitude 21m), Brazil.

We aimed to review the clinical, epidemiological and pathological aspects of the clostridial diseases diagnosed in cattle from January 1978 to December 2018 at the LRD-UFPel in the hopes that it will constitute a useful guide for field veterinary practitioners and interested farmers.

MATERIALS AND METHODS

We reviewed the necropsy protocols of 6,736 necropsies of cattle performed from January 1978 to December 2018 and filed at the LRD-UFPel. These necropsies were performed either by LRD-UFPel faculty or by field veterinary practitioners from the LRD-UFPel influence area, who subsequently submitted material for histological evaluation at the LRD-UFPel. We selected cases of clostridial diseases found in these 6,736 necropsies. From each outbreak, we annotated the following data: municipality of origin, year of occurrence, age of affected cattle, type of breeding, morbidity, mortality and lethality rates. The authors did not make any changes in the final diagnosis that appeared in each protocol.

We also made a brief review on the epidemiology, clinical signs, necropsy findings, histopathology and methods of diagnosis and prophylaxis of each clostridial disease reported in this study.

RESULTS AND ADISCUSSION

From the results of the 6,736 bovine necropsies reviewed here, 1,000 (14.85%) were of bacterial diseases. A hundred and four of those (11.1%) were clostridial diseases. In this review, out of a total of 42,480 cattle at risk, approximately 904 died, reaching a mortality rate of 2.13%. Five specific clostridial diseases were found. In descending order of frequency, there were 35 outbreaks of tetanus (31.5% of the clostridial disease), 34 outbreaks of blackleg (30.6% of the cases), 23 outbreaks of bacillary hemoglobinuria (20.7% of the cases), 11 outbreaks of malignant edema (gas gangrene) (9.9% of the cases), and 8 outbreaks of botulism (7.2% of the cases). Epidemiological data on the outbreaks of each disease are in Table 1. With the exception of botulism, in all clostridial diseases outbreaks of this study, a lethality rate of approximately 100% was observed, which shows the importance of this category of diseases as causes of cattle losses.

There was an unusual frequency of cases of bovine tetanus, especially if we consider that cattle is relatively resistant to the disease (Popoff 2016) when compared for example, with horses, and that the disease tends to be sporadic in cattle (Raposo 2007). However the following unusual epidemiological conditions explain the high incidence of tetanus in this study: in 2001 large outbreaks of tetanus occurred in five farms in the area of influence of the LRD-UFPel. In these outbreaks at least 297 beef cattle and 50 sheep died. Injections with an anthelmintic contaminated with *Clostridium tetani* was applied to a large number of cattle and caused unusually the large outbreaks (Driemeier et al. 2007). In 2009, 24 outbreaks of tetanus occurred in the southern RS. In this particular outbreak there was a history of drug and/or vaccine application in most of the herds (Quevedo et al. 2011).

Tetanus is a neurological disease characterized by spastic paralysis. It results from the contamination of wounds with spores of *Clostridium tetani* acquired from the environment (Popoff 2016). Deep wounds with little exposure to air and the presence of necrotic tissue provide anaerobic conditions favoring spore germination (Popoff 2016).

Spastic paralysis results from the action of TeNT produced by *C. tetani* at the synapses of the interneurons in the central nervous system. When the TeNT reaches the neuromuscular end plate, becomes internalized at the presynaptic terminal (Mayhew 2009). Through retrograde transport (Bizzini 1989), it reaches the body of the motor neuron, from where it is transferred to the inhibitory neurons within the spinal cord and brain stem (Fig.1). TeNT compromises the release of neurotransmitter at the synapse, preventing the inhibitory neuron from disrupting the action triggered by the motor neuron, then resulting in spasticity (Barros et al. 2006b, Mayhew 2009).

The potential risk for tetanus in cattle exists in almost all rural properties due to the ubiquitous occurrence of *C. tetani* in the soil, water, and feces of animals and human beings, and poor husbandry practices that create favorable conditions for

Table 1. Epidemiological data on the five outbreaks of clostridial diseases diagnosed in cattle by the "Laboratório Regional de Diagnóstico" of the "Universidade Federal de Pelotas" from 1978 to 2018

Clostridial diseases	Season	Number of outbreaks per season	Total number of outbreaks	Clatle at risk	Maximum-minimum morbidity (%)	Maximum-minimum mortality (%)
Tetanus	Spring	10				
	Summer	3	35	18.942	0,2-39,5	0,2-39,5
	Fall	9				
	Winter	13				
Blackleg	Spring	9				
	Summer	3				
	Fall	7	34	7,297	0,06-50	0,06-50
	Winter	15				
Bacillary hemoglobinuria	Spring	7				
	Summer	3	23	12,792	0,1-17,3	0,1-17,3
	Fall	6				
	Winter	7				
Maligntant edema	Spring	3				
	Summer	6	11	714	0,62-100	0,62-100
	Fall	1				
	Winter	1				
Botulism	Spring	1				
	Summer	5	8	2.735	0,5-10,8	0,16-10,8
	Fall	2				
	Winter	0				

Lethality rates were virtually 100% in all outbreaks except for botulism in which lethality veraged 30%.

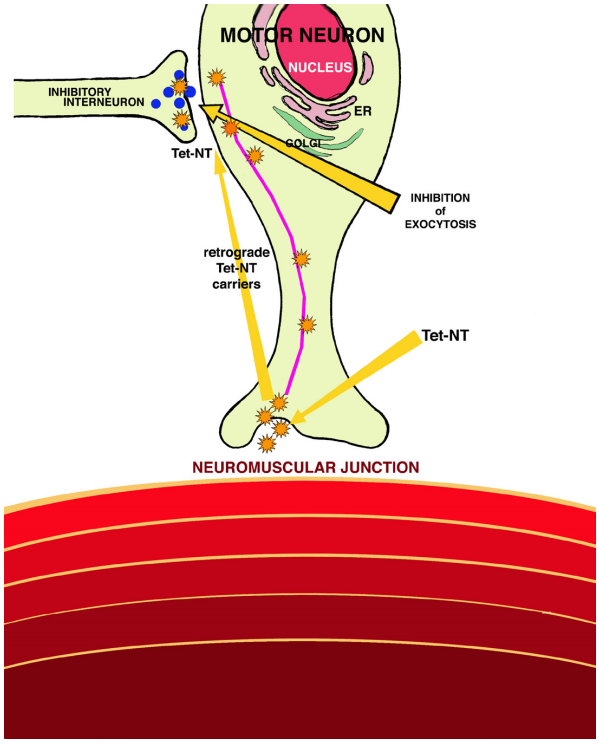


Fig.1. Pathogenesis of tetanus. Tetanus neurotoxin (TetNT, yellow asterisks) is directed by retrograde transport (shown as a purple line) into motor neurons by the action of microtubules and actin microfilaments. TetNT inhibits the exocytosis of specific synaptic vesicles at inhibitory neuronal terminals. Thus the action of the inhibitory neuron is not exerted on the motor neuron, resulting in spasticity. (Modified from Mayhew 2009)

the development of the disease. The port of entry is usually a perforating or cutting wound, contaminated by soil, feces or other material containing bacterial spores and anaerobic microorganisms. These wounds may be related to castration, umbilical stump, calving (Odendaal & Kriek 2004) or may be caused by scarification of the intestinal mucosa by plant fibers (Raposo 2007). The bacterium has limited capacity to invade the host, restricting itself to the inoculation site (Raposo 2007).

In Brazil, the occurrence of tetanus is generally sporadic but, as was already mentioned outbreaks with high morbidity, mortality and lethality have occurred in cattle handled in pens for some kind of procedure, the use of unhygienic equipment or contamination of the skin by dust or mud during vaccination and de-worming practices (Salvador & Freire 1998, Barros et al. 2006b, Driemeier et al. 2007).

The incubation period is 18 hours to 4 weeks in most cases 7-15 days (Driemeier et al. 2007). The lower the incubation period and progression, the more severe is tetanus. There is also a correlation with the port of entry: lesions close to the head usually result in more severe disease. In cases with a long incubation period, it may not be possible to detect the portal of entry (Barros et al. 2006b).

In cattle, early signs include mild or moderate bloat tremors, mandibular trismus, muscular rigidity (Fig.2) and prolapse of the third eyelid and opisthotonus (Fig.3). Affected cattle are alert and anxious with erect ears. Asymmetric contractions induce spinal curvature and lateral deviation of the tail (Bleck 1989, Driemeier et al. 2007).

No specific morphological changes are observed. Gray matter hemorrhages, decubitus pneumonia and asphyxia lesions reported by some authors (Valette & Petermann 1988)

are secondary changes or artifacts. The diagnosis of tetanus is primarily clinical and epidemiological. Prophylaxis includes the application of two doses of vaccine with an interval of four weeks between applications.

Of the 34 outbreaks or sporadic cases of blackleg diagnosed in cattle, 15 occurred in the winter, nine in the spring, seven in the fall, and three in the summer. As has already been observed (Heckler et al. 2018) blackleg cases tend to be distributed throughout the year without showing specific seasonality (Table 1). However, it is also described that the prevalence of the disease was higher during the fall (Riet-Correa 2007a). The age of affected cattle in this study ranged from four months to two years and exceptionally, in one outbreak a 4-year old cow was affected. Usually 6-24 month-old well nourish cattle are affected (Useh et al. 2006), but cases were already reported in a 3-day calf (Sojka et al. 1992) and a bovine fetus transplacentally infected (Abreu et al. 2017). In Mato Grosso do Sul state the disease has been diagnosed in cattle of 2.5-3 years old, not vaccinated or vaccinated long time before the outbreak (Riet-Correa 2007a, Heckler et al. 2018).



Fig.2. Clostridial diseases. Tetanus. Cow with spastic paralysis.



Fig.3. Clostridial diseases. Tetanus. Steer with opisthotonus.

Roughly our data are similar. Surveys of the prevalence of clostridial diseases in Brazil generally indicate blackleg as the most frequent disease (Correa et al. 1980, Riet-Correa 2007a, Heckler et al. 2018). In our study, this is also a trend. However for reasons already explained there were more cases of tetanus, which is unusual.

The pathogenesis of blackleg is relatively well established (Useh et al. 2006, Abreu & Uzal 2016, Abreu et al. 2017). Spores of *Clostridium chauvoei* are ingested from the contaminated soil and absorbed by the intestinal mucosa gaining access to the blood circulation. The spores are then distributed through various tissues, mainly skeletal muscles and myocardium, where they become dormant. When the occurrence of an injury (which can be trauma, injection, etc.) favors a local anaerobic environment, spore germination occurs and the bacteria produce potent exotoxins that cause necrotizing myositis. The proliferating bacteria and toxins then enter the bloodstream causing exotoxemia and death (Fig.4).

The clinical manifestations of blackleg are often not observed. Due to the rapid clinical course, cattle are often found dead; sudden death may occur and are generally attributed to myocardial lesions (Williams 1977, Glastonbury et al. 1988, Helman et al. 1997, Uzal et al. 2003). The clinical picture is acute or hyperacute and clinical signs consists of lameness, swelling, muscle crepitation and fever. Death invariably occurs within 24 to 36 hours from the onset of the clinical signs (Barros 2016b).

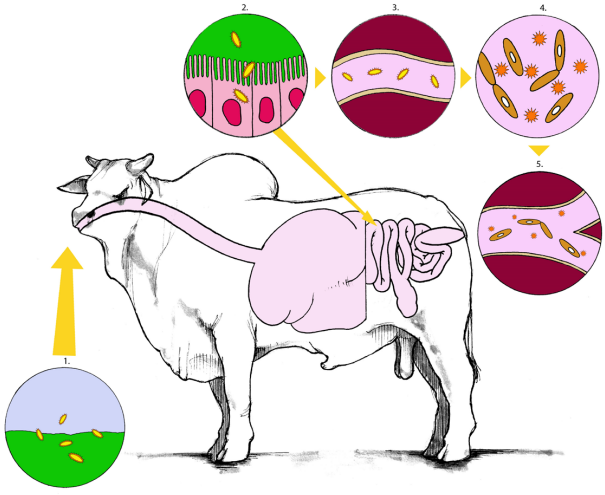


Fig.4. Blackleg. Pathogenesis. *Clostridium chauvoei* spores are ingested from the contaminated soil (1) and absorbed through the intestinal mucosa into the blood circulation (2). The spores are then distributed through various tissues, mainly skeletal muscles, where they become dormant within macrophages (3). When a muscle injury occurs, a favorable anaerobic environment is created, the spores germinate, and the bacteria produce potent exotoxins that cause hemorrhagic necrotizing emphysematous myositis (4). The proliferating bacteria and toxins enter the bloodstream causing exotoxemia and death (5) (Modified from Abreu et al. 2017).

The cadaver of a bovine that dies from blackleg quickly swells. Significant macroscopic changes include localized swelling and crepitus of the muscles in the hind limbs and chest (Barros 2016b). On the cutting surface, the affected muscle is dark-red and contains numerous gas bubbles (Fig.5). In the periphery, the muscle is red and moist due to edema (early lesions). At the center of the lesion, the affected muscle is dark-red, dry and friable (mature lesions) (Barros 2016b, Heckler et al. 2018). Muscle affected by blackleg often has a butyric odor, such as rancid butter (Cooper & Valentine 2016). In addition to skeletal muscle, heart and tongue may be affected (Fig.6). Myocardial lesions are similar to those of skeletal muscles and associated with fibrinous pericarditis and pleuritis (Williams 1977, Abreu & Uzal 2016). Histologically, necrotic fibers appear dissociated by gas bubbles and hemorrhage (Fig.7), and there is meager neutrophilic infiltrate (Barros 2016b). In some cases, one can observe intralesional gram-positive rods. Vaccination is the preferred method of control (Abreu & Uzal 2016)

The results of this survey demonstrate that the most critical clostridial diseases in cattle in the southern area of Rio Grande do Sul (RS) state are blackleg and bacillary hemoglobinuria (BH), with 34 and 23 outbreaks respectively. To make this assumption one have to consider the conditions in which many tetanus outbreaks occurred in the period of the current study, as explained previously. In the outbreaks of BH the morbidity, mortality and lethality ratios were the same (Table 1).

BH is an acute or subacute disease, usually fatal, affecting mainly cattle and, more rarely, other species (Schild 2007). It is caused by toxins of *Clostridium haemolyticum* referred to, also by some authors as *Clostridium novyi* type D (Kriek & Odendaal 2004), a rod, sporulated, gram-negative anaerobic (Schild 2007).

BH may be sporadic or endemic. Morbidity is 0.25%-12% (Schild 2007) and the mortality is 80-100% (Navarro et al. 2016). High incidence of BH occurs in flooded regions where the occurrence of hepatic trematodes, mainly *Fasciola hepatica* exist (Barros 2016a). In other countries *Fascioloides magna* and *Dicrocoelium dendriticum* may also be associated with the disease (Navarro et al. 2016). The main determinant of the incidence of the disease is the amount of hepatic trematodes in the cattle grazing area (Navarro et al. 2016). However in some cases of the outbreaks reported in this study, no evidence of parasitism was observed in the liver. This might indicate that the parasitic lesions where masqueraded or that other factors may be involved in triggering the lesions. Liver biopsy was already inculpated as one of these factors (Monaghan & Sheahan 1987), but is improbable that cattle in these outbreaks had been submitted to this procedure.

In the current study BH was the second most frequent clostridial disease observed in four municipalities in RS, all of which had low terrains and soils used mainly for planting rice. In these regions there are snails of the genus *Lymnea* which are intermediate hosts for *F. hepatica*. Accordingly in the current study BH occurred throughout all seasons of the year and all outbreaks were observed in municipalities in the southern part of the state, where there is flat and low lands mainly used for rice crops. *F. hepatica* can cause liver lesions that determine an anaerobic environment favoring multiplication of *C. haemotyticum*.



Fig.5. Clostridial diseases. Blackleg. Gross lesions at the hidlimb muscles. The muscle to the right is moist (early lesion). The dark, dry muscle to the left represents a more advanced phase of the lesion. Observe the well-developed gas bubbles in the muscle at the top right.

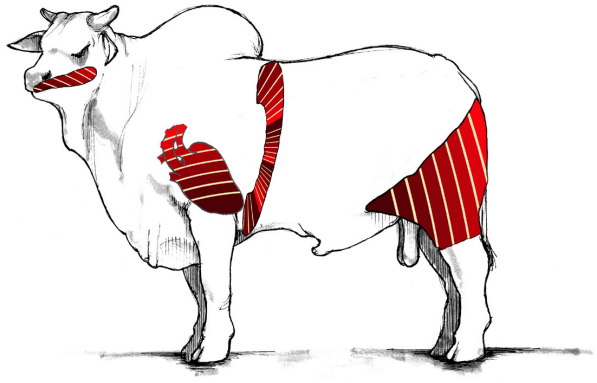


Fig.6. Clostridial diseases. Blackleg. Schematic representation of the anatomical sites where muscle lesions are more commonly seen: muscles of the thigh, diaphragm, heart, and tongue.

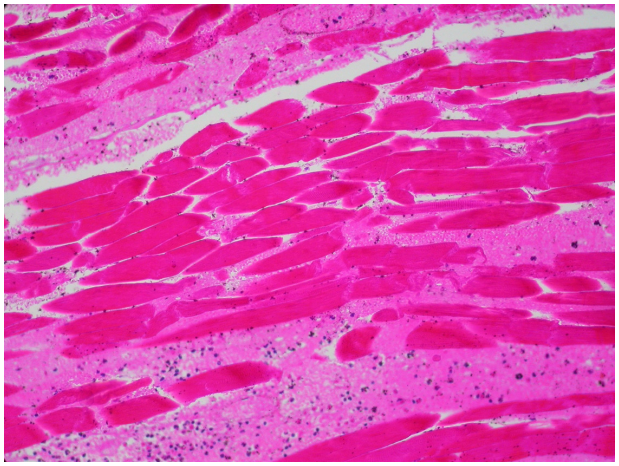


Fig.7. Clostridial diseases. Blackleg. Hemorrhagic necrotizing myositis. A histopathological aspect of the striated muscle lesion. There is hyaline necrosis of myofibers and edema and hemorrhage between myofibers. HE, obj.40x.

In RS, BH affects well-nourished cattle older than two years pasturing in low fields such as those on the coast (Schild 2007, Barros 2016a). Outbreaks of the disease have also been observed after periods of flood when conditions for increasing the population of *F. hepatica* producing overwhelming numbers of metacercariae (Schild 2007). In these cases BH may become endemic. Paradoxically, in these regions, dry conditions induce cattle to concentrate in parts of the field where there is some water and consequently high populations of *F. hepatica* (Navarro et al. 2016).

Affected cattle can eliminate spores through feces and urine, thus contaminating pastures. Although it is described as a major occurrence in the summer and autumn (Schild 2007), in our study BH occurred with the same frequency throughout the year.

Essential aspects of pathogenesis are as follows (Kriek & Odendaal 2004): as previously state BH occurs only in regions where both *F. hepatica* and *C. haemolyticum* are endemic. In the life cycle (Fig.8) of *F. hepatica* the final host ingests the metacercariae which are attached to the grass (Tessele et al. 2013). The ingested metacercariae penetrate the duodenum of the definitive host, cross the intestinal wall, migrate through the coelom and penetrate the capsule of the liver, migrating through the hepatic parenchyma to reach the hepatic ducts. The migration of the metacercariae through the liver harboring latent *C. haemolyticum* spores (within Kuppfer cells) induces hepatic lesions (Fig.9) and those create the necessary conditions for bacterial growth and toxin production (Kriek & Odendaal 2004).

BH is a hemolytic disease and clinical signs are largely the result of intravascular hemolysis (Barros 2016a). The disease may be acute (clinical course of 10-12 hours) or subacute (3-4 days), but under grazing conditions cattle are generally found dead (Navarro et al. 2016). The incubation period is 7-10 days (Barros 2016a).

Clinical signs include hemoglobinuria, jaundice and fever (40-41°C), that tends to disappear with the progression of the disease, and blood in the feces (Kriek & Odendaal 2004,

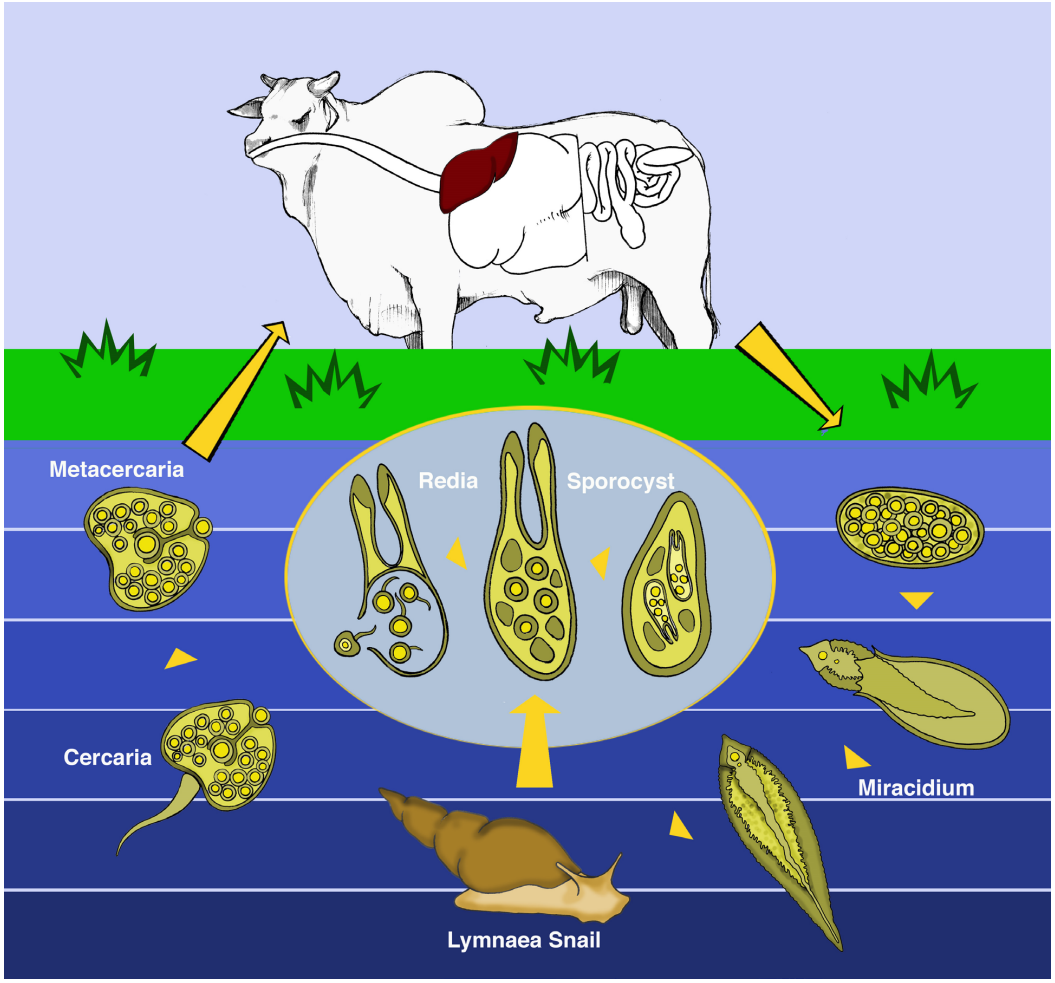


Fig.8. The life cycle of *Fasciola hepatica*. Eggs are shed in the bile and feces. A hatching egg releases the miracidium that survives only in humid environments. The miracidium penetrates actively in the intermediate host (snail of the genus *Lymnaea*). Inside the snail, each miracidium gives rise 5-8 rediae which originate daughter rediae and cercariae. Cercariae leave the snail and set themselves on grass, just below the water level, turning into metacercariae. The final host ingests the metacercariae along with the grass. People and other domestic and wild mammals can also become infected. The ingested metacercariae cross the duodenum of the definitive host and penetrate the intestinal wall, wander through the coelom, penetrate the capsule of the liver, and migrate through the hepatic parenchyma to reach the hepatic ducts (Modified from Tessele et al. 2013).

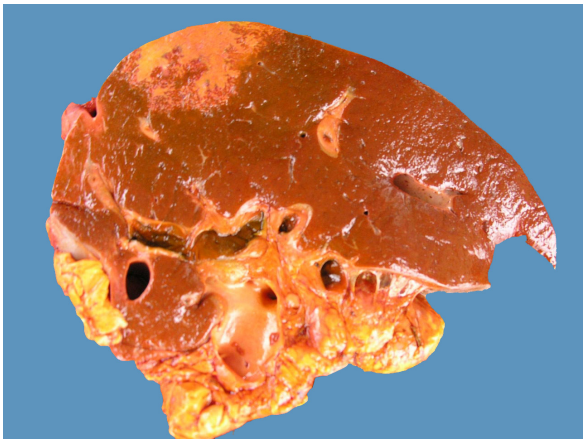


Fig.9. Clostridial diseases. Bacillary hemoglobinuria (BH). Liver, cut surface. The triangular single white area of coagulative necrosis seen to the right is a hallmark lesion of BH. The ducts are thick walled (chronic cholangitis) and some specimens of *Fasciola hepatica* are in their lumen. Migration of *F. hepatica* metacercariae through liver parenchyma favors sporulation of *Clostridium haemolyticum*, the causative agent of BH.

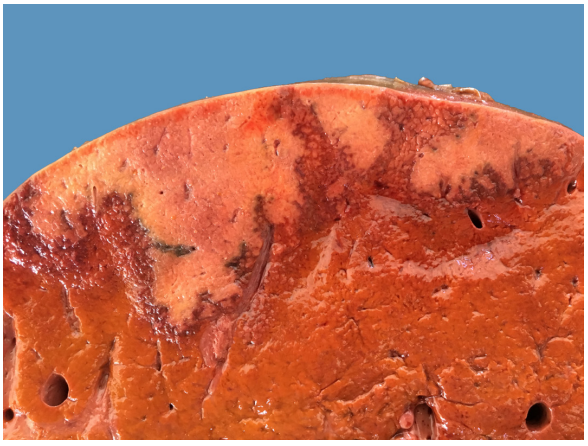


Fig.10. Clostridial diseases. Bacillary hemoglobinuria. Liver, cut surface. Notice the large irregular bordered pale area of coagulative necrosis in the liver parenchyma. A red rim of hemorrhage and inflammation surrounds the necrotic focus.

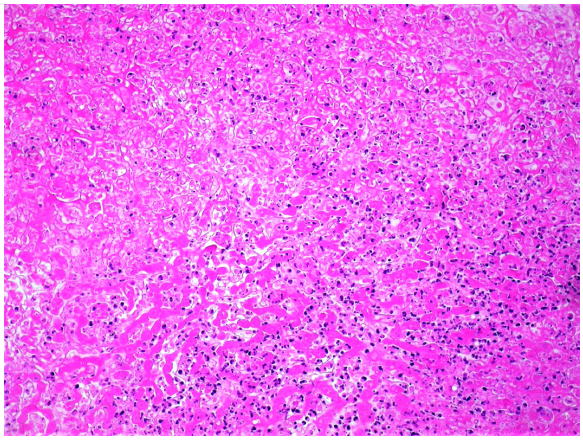


Fig. 11. Clostridial diseases. Bacillary hemoglobinuria. Histopathology. A large area of coagulative necrosis to the right is surrounded by a rim of leucocytes. HE, obj.20x.

Schild 2007, Barros 2016a, Navarro et al. 2016). Jaundice may not be very striking. Pregnant cows may abort. There is anemia (red blood cells 1-4 x 106/mm^3 and hemoglobin 3-89mg/dl and leukogram 7,700-34,800 leukocytes/mm^3) and glycemia can be 100-200mg/dl in some cases (Schild 2007, Barros 2016a, Navarro et al. 2016).

The main necropsy findings are single 5-20cm white to tan necrotic areas in the hepatic parenchyma which is orange due to pigmentation by bilirubin (Barros 2016a). Such areas (Fig.10) may be ischemic resulting from thrombosis or may be the direct action of toxin (Barros 2016a, Navarro et al. 2016), or both. These areas of necrosis are considered pathognomonic when observed in conjunction with other clinical signs (Navarro et al. 2016). Other findings are the same as in any intravascular hemolytic disease and include jaundice, petechial hemorrhages in the subcutaneous tissue, dark kidneys, and coffee-colored urine (Schild 2007).

Histopathologically, the area of coagulative necrosis is surrounded by an inflammatory border of neutrophils (Fig.11) and intralesional rods can be found within the liver sinusoids. Hemoglobinuric nephrosis are usually observed (Navarro et al. 2016).

The diagnosis is based on the epidemiology, characteristic necropsy findings, and can be confirmed by polymerase chain reaction, fluorescent antibody technique and immunohistochemistry. The indicated form of control is vaccination (Navarro et al. 2016).

We found eleven outbreaks of malignant edema (gas gangrene) in our survey. Interestingly in one of those, there was 100% of lethality induced by poor husbandry. Alternatively, good husbandry can prevent this clostridial disease.

Malignant edema is a clostridial infection that mainly affects ruminants (Odani et al. 2009), horses (Peek et al. 2003, Raymundo et al. 2010, Farias et al. 2014) and other mammals and birds (Silva et al. 2016b). It is due to contamination of wounds by bacteria of the genus *Clostridium* including one or more of the following: *Clostridium septicum*, *Clostridium chauvoei*, *Clostridium novyi* type A, *Clostridium perfringens* type A, and *Clostridium sordellii* (Prescott 2016, Silva et al. 2016b). All of these microorganisms are ubiquitous and can be found in the environment and the intestines of animals and humans (Silva et al. 2016a).

Many authors consider malignant edema and gas gangrene as synonyms (Silva et al. 2016b, Riet-Correa 2007a). Others separate them into two distinct diseases (Cooper & Valentine 2016) with the following explanation for the split: both diseases are associated with subcutaneous and muscular hemorrhagic edema and necrosis; when gas is produced in the lesion the term gas gangrene is appropriated. In the absence of gas and when edema predominates, the disease is referred as malignant edema. According to these authors (Cooper & Valentine 2016) *C. septicum* would be a more common cause of gas gangrene, and *C. perfringens* of malignant edema.

In this paper we consider malignant edema and gas gangrene as synonyms and we favor the use of the term "malignant edema" for the convenience of a single denomination for these conditions and because, in our experience, hemorrhagic edema in the subcutaneous tissue and between the muscles is the most frequent lesion at necropsy; more rarely, lesions occur in the muscles (myonecrosis). The typical lesion is acute cellulitis. An identical clostridial disease induced by *C. novyi*

type A is observed in rams which, to increase confusion, is known yet by another sobriquet: "swollen head" (Cooper & Valentine 2016, Prescott 2016).

Cases of malignant edema are observed sporadically. Outbreaks occur when there is collective trauma. It affects animals of any age. It can occur after dipping, shearing, tail docking, castration, deliverance (Odani et al. 2009), or injections with contaminated needles. When the disease is caused by the use of contaminated needles, mortality is very high in the first 48 hours, as occurred in an outbreak observed in this study, where outbreaks caused by syringes contaminated with *C. septicum* caused 100% mortality in calves. In Mato Grosso do Sul, outbreaks of malignant edema with tongue myonecrosis were apparently caused by contamination of wounds caused by grazing coarse grass (Lemos 1998).

For the diagnosis of malignant edema, one must search for recent injuries to the skin (which does not appear in blackleg) and for the characteristic necropsy lesions. The identification of the agent can be performed by immunofluorescence, immunohistochemistry (Assis et al. 2005), or isolation and characterization of the *Clostridium* spp. (Riet-Correa 2007b) and type. In young cattle (6 months to 2 years old) the disease should be differentiated from blackleg. In blackleg, there is no association with wounds and muscular lesions always predominate.

Affected animals may be treated with high doses of penicillin or broad-spectrum antibiotics. For prophylaxis, it is necessary to avoid contamination, especially with soil, of the instruments and syringes used in the herd. The animals should be vaccinated annually with vaccines containing mainly *C. septicum*, *C. novyi* type B, and *C. sordelli*.

In seven out of the eight botulism outbreaks (7.7% of all clostridial diseases), in the current study, cattle were in a pasture of native grass, and in only one outbreak cattle were supplemented with minerals. The majority of cases (5/8) occurred in the municipality of Rio Grande, RS, in flooded areas, which have not being used for crops since they were submerged for the most part of the year. In three outbreaks there was animal carrion in the pasture. Five outbreaks occurred in the summer (January-February), two in the fall and one in the spring. Morbidity, mortality and lethality were from 0.5-10.8 to 0.16-10.8 respectively and there was an average of 30% lethality (Table 1).

Botulism is a clostridial disease whose main characteristic is flaccid paralysis of various muscle groups. It is usually caused by the ingestion of botulinum neurotoxins (BoNTs) produced by *Clostridium botulinum* (an anaerobic gram-positive bacterium) found in the soil, water, food, and alimentary system of many animals (Le Marechal et al. 2016).

There are seven potent distinct BoNTs identified by the letters A-G (Dover et al. 2014, Maslanka et al. 2016). BoNTs are the most toxic biological substances that exist (Lamanna 1959); a gram of pure toxin evenly distributed in the feed can kill 400,000 adult cows (Galey et al. 2000). After ingestion and absorption from the intestine, BoNT reaches the bloodstream, is disseminated in the body and exerts its effect on the peripheral somatic motor nerves that supply skeletal muscle. At the neuromuscular junction, the neurotoxin inhibits the exocytosis of acetylcholine, which compromises the stimulus for muscle contraction, resulting in flaccid paralysis, a hallmark of the disease (Fig.12). The central nervous system is spared because it is protected by the blood-brain barrier (Mayhew 2009). There are no gross or histologic lesions associated with botulism (Barros et al. 2006a).

Botulism is endemic in Brazil, mainly in the Midwestern states (Soares et al. 2018) and the BoNTs responsible for the outbreaks in cattle in our country are, mostly, types C and D (Barros et al. 2006a, Silva et al. 2016). In the last 15-20 years, botulism has emerged as a significant disease in Europe and a bioterrorism threat (Skarin et al. 2013).

Cattle become ingesting the BoNTs from carrion, drinking stagnant water contaminated with animal carrion (Dutra et al. 2001), degraded food, or food contaminated with carrion from rodent, birds, and cats (Galey et al. 2000).

In cattle, the morbidity is variable, and the lethality is generally 100%, although there are cases of recovery of cattle that ingest small doses and develop mild clinical signs (Barros et al. 2006a, Constable et al. 2017).

In an outbreak in Paraíba state, northeastern Brazil, 96.66% of sheep (145/150), 51.77% of goats (233\450), and 96.77% of cattle (30\31) died after the ingestion of chicken

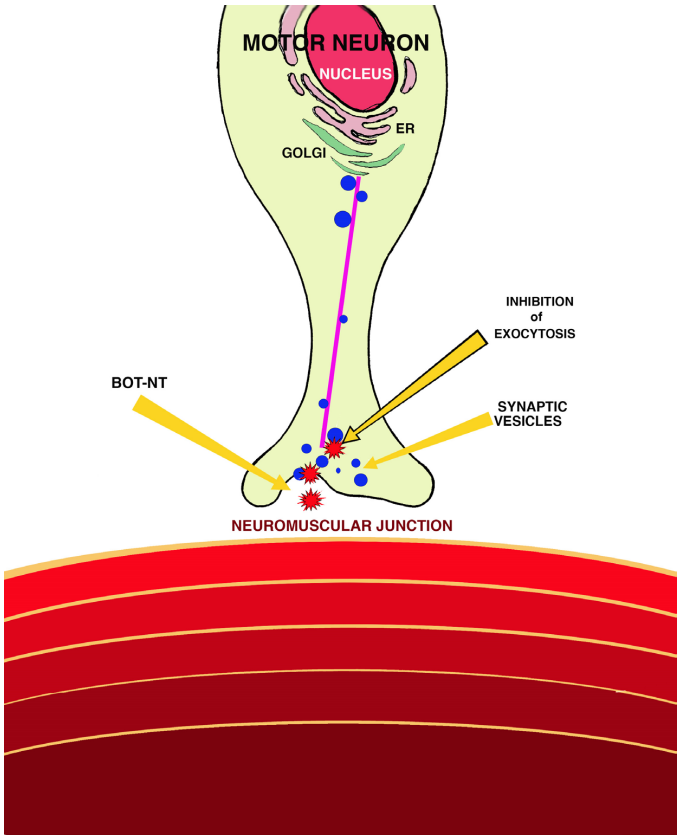


Fig.12. Pathogenesis of botulism. Schematic view of a motor neuron and the neuromuscular junction, The botulinum neurotoxin (BoNTs, shown as red asterisks) are internalized at synapses in endosomal compartments, one of which might attach to synaptic vesicles (blue dots). At the neuromuscular junction, the BoNT inhibits the exocytosis of acetylcholine, which compromises the stimulus for muscle contraction, resulting in flaccid paralysis. The synaptic vesicles are synthesized in the endoplasmic reticulum and released by the Golgi apparatus. (Modified from Mayhew 2009)

litter (Riet-Correa et al. 2003). In a feedlot of 1,700 cattle Mato Grosso do Sul, 1,090 steers died within four days (Guizelini et al. 2019).

Clinical signs of botulism may appear 1-17 days after ingestion of contaminated food (Barros et al. 2006a, Constable et al. 2017). Although most cases are acute, the course of the disease may be hyperacute (less than 24 hours), acute (1-2 days), subacute (3-7 days), or chronic (7-30 days). In the chronic form affected cattle have better chances of survival (Barros et al. 2006a). It is likely that the dose of toxin ingested determines the course of the disease. Larger doses would determine acute conditions, while lower doses would cause chronic disease.

The clinical signs of botulism are quite characteristic so much so as to allow a reliable clinical diagnosis (Barros et al. 2006a). It is characterized by partial or complete flaccid paralysis of the muscles of locomotion, chewing and swallowing. The animals present a decrease, but never a complete absence, of the musculature of the limbs, with flaccid paralysis of two (Fig.13) or four limbs.



Fig. 13. Clostridial diseases. Botulism. Flaccid paralysis in a cow. The lack of tonus in the muscle limbs (flaccidity) prevents the steer from standing.



Fig. 14. Clostridial diseases. Botulism. Flaccid paralysis causes the tongue to protrude from the mouth.

The main clinical sign is the difficulty of locomotion, characterized by staggering gait, affecting mainly the hind limbs and evolving to the forelimbs, head and neck. The animal tends to lie in sternal decubitus with the head resting on the flank or with the chin on the ground. Flaccid paralysis affects the tongue (Fig.14) which is easily pulled from the mouth. There is also flaccidity of the tail, which remains the medial and distal portions apart from the body. Bradycardia occurs, and respiration is dyspneic, laborious, diaphragmatic (abdominal), with inspiration in two phases, the second being prolonged. There is flaccidity of chewing muscles, which is observed as inability to seize, chew and swallow the food (Barros et al. 2006a).

Rarely, there are sensory abnormalities, which can be assessed by maintaining cutaneous, paravertebral and limb sensitivity. While cattle lied down for prolonged periods, they may develop ischemia of large muscle masses. In this case, there will be a loss of sensitivity due to muscle and nerve compression (Barros et al. 2006a)

The diagnosis of the disease is based on the clinical signs, the history, and the absence of significant gross and histological lesions. Botulism should be suspected whenever cattle in good condition, are found in a permanent position. As botulism is a neurological peripheral disease there are no obvious changes in attitude and sensibility. Botulism usually affects cattle of various ages spectrum.

Other techniques can be employed to confirm a clinical diagnosis. Intraperitoneal inoculation in mice (biological assay) of 500ml of hepatic extract, blood serum, ruminal or intestinal contents is considered the most specific test but still has a very low sensitivity. When the test is positive, one should perform the serum neutralization test (or seroprotection), which is based on the neutralization of the botulinum toxin with the specific antitoxin.

Treatment for botulism in cattle is controversial, but some limited results have been obtained with hyperimmune serum (Guizelini et al. 2019). As an alternative, supportive treatment should be performed (Barros et al. 2006a). The elimination of field carcasses is an important auxiliary measure. Vaccination of the herd is another important form of control and prophylaxis in areas where there is a high incidence of the disease. Animals can be vaccinated from four months of age and revaccinated within 30-40 days, and then annually.

Some final considerations are in order. The five clostridial diseases discussed here have characteristic clinical signs and epidemiological features. Three of them have necropsy findings and histopathology so characteristic that, when associated with clinical signs epidemiology, allow for a diagnosis. Two others do not present anatomopathological findings, but have almost unmistakable clinical signs (Table 2) and laboratory diagnostic techniques that might confirm them are suboptimal or nonexistent. In no way are we advocating against the use of appropriate laboratory diagnostic techniques. These considerations are made to explain that, even although, in the current study we made the diagnoses without the aid of such ancillary techniques, the diagnosis are sound. We admit that a few diagnostic errors may have been committed, but not to the point of damaging the trend of the data provided here. In Table 2, we suggest and approach for a field working diagnosis of these diseases.

Table 2. Approach for the diagnosis of some clostridial diseases

Disease	*Clostridium* sp.	Clues for the diagnosis			
		Epidemiology	Clinical signs	Gross lesions	Histopathology
Tetanus	*C. tetani*	Skin wounds	Spastic paralysis, prolapse of the third eylid, acute course	None	None
Blackleg	*C. chauvoei*	Blunt rauma	Stiffness of the limbs, claudication. Crepitation of affected area. Usually found dead	Hemorrhagic necrotizing enphysematous myositis, same lesion in the myocardium; fibrinous pericarditis, and pleuritis	Hemorrhagic necrotizing enphysematous myositis
Bacillary hemoglobinuria	*C. haemolyticum*	Pastures with bodies of water, *Fasciola hepatica*	Jaundice, red urine, bloody liquid feces, fever, acute course	Single large necrotic área in the liver. Red urine. Dark kidneys.	Liver necrosis with intralesional rods
Malignant edema	*C. novyi* type A, *C. perfringens* type A, *C. sordelli, C. septicum*	Skin wound	Swelling, heat and pain in affected área. Acute disease. Sometimes crepitation of affected area.	Hemorrhagic subcutaneous edema, Less frequently emphysematous myonecrosis.	Subcutaneous edema, Necrotic myositis.
Botulism	*C. botulinum*	Carrion in the food, and pasture, stagnant water contaminated by carrion, degraded food, poultry litter	Flaccid paralysis,	None	None

CONCLUSIONS

Five clostridial diseases in cattle should be considered in the field diagnosis in southern Rio Grande do Sul, namely tetanus, blackleg, bacillary hemoglobinuria, malignant edema, and botulism.

Certain characteristic clinical, epidemiological, necropsy and histopathological findings (or instead, their absence) are fundamental for the diagnosis in the field.

Treatment is possible but, in most cases, unsuccessful due to the acute course of these five diseases. Vaccination is the best procedure to prevent any one of them.

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Original Article
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Spontaneous and experimental poisoning by *Merremia macrocalyx* (Convolvulaceae) in cattle[1]

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ABSTRACT.- Brito L.B., Silva Filho G.B., Chaves H.A.S., Nascimento A.L.O., Braga T.C., Pfister J., Riet Correa F. & Mendonça F.S. 2019. **Spontaneous and experimental poisoning by *Merremia macrocalyx* (Convolvulaceae) in cattle.** *Pesquisa Veterinária Brasileira 39(7):447-453.* Laboratório de Diagnóstico Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manoel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: mendoncafs@yahoo.com.br

The aim of this work was to describe the epidemiological, clinical and pathological aspects of spontaneous poisoning by *Merremia macrocalyx* in cattle in the Pernambuco state, northeastern Brazil, and to experimentally replicate the poisoning by this plant. To determine the occurrence of poisonings, 30 farms were visited in six municipalities at the Forest Zone of Pernambuco. The plant was found in nine farms, in which history of plant poisoning in cattle, and occasionally in sheep were also reported. Three outbreaks of spontaneous poisonings in cattle were studied. To replicate the disease experimentally, two steers received a single dose of 60g/kg and two steers received 80g/kg of the fresh leaves of *M. macrocalyx* in the trough for spontaneous ingestion. Two steers were also used as a control group. The main clinical signs observed in spontaneous cases consisted of restlessness, bloat, polyuria, diarrhea, and death within 48 to 72 hours after the onset of clinical signs. Cattle experimentally poisoned presented similar clinical signs to those observed in spontaneous cases. Gross lesions consisted of dryness and impaction of the rumen, omasum and reticulum contents. Abomasal content was fluid, the mucosa was hyperemic, with swollen folds and multiple ulcers. Similar lesions were observed in duodenum mucosae. Histologically, lesions observed in the abomasum and duodenum mucosa consisted of necrosis, hemorrhage and inflammatory infiltration of neutrophils and lymphocytes. The wide distribution and palatability of this plant, associated with the high sensitivity of the bovine species verified in this experiment, highlight the importance of this plant in spontaneous cases of poisoning in cattle.

INDEX TERMS: Spontaneous poisoning, experimental poisoning, *Merremia macrocalyx*, Convolvulaceae, toxic plants, plant poisoning, tympanism, ruminants, Pernambuco, Brazil, cattle, toxicoses.

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RESUMO.- [**Intoxicação espontânea e experimental por *Merremia macrocalyx* (Convolvulaceae) em bovinos na Zona da Mata de Pernambuco.**] O objetivo deste trabalho é descrever os aspectos epidemiológicos, clínicos e patológicos da intoxicação espontânea por *Merremia macrocalyx* em bovinos de Pernambuco, nordeste do Brasil e reproduzir experimentalmente a intoxicação por esta planta. Para determinar a ocorrência das intoxicações, foram visitadas 30 propriedades em seis municípios na Zona da Mata de Pernambuco. A planta foi encontrada em nove fazendas

onde também haviam históricos de intoxicações em bovinos e ocasionalmente em ovinos. Três surtos de intoxicações espontâneas em bovinos foram estudados e para reproduzir experimentalmente a doença, dois novilhos receberam doses únicas de 60g/kg e dois novilhos receberam 80g/kg de folhas frescas de *M. Macrocalyx* para consumo espontâneo no cocho. Dois novilhos foram utilizados como grupo controle. Os principais sinais clínicos observados na intoxicação espontânea consistiram em agitação, timpanismo, poliúria, diarreia e morte dentro de 48 a 72 horas após a observação dos primeiros sinais clínicos. Os bovinos intoxicados experimentalmente apresentaram sinais clínicos semelhantes aos observados nos casos espontâneos. À necropsia as lesões consistiam em compactação e ressecamento dos conteúdos do rúmen, omaso e retículo. O conteúdo do abomaso estava fluido, notava-se hiperemia das mucosas, as pregas estavam edemaciadas e continham múltiplas úlceras. Lesões semelhantes também foram observadas na mucosa do duodeno. Histologicamente, as lesões observadas na mucosa do abomaso e do duodeno consistiam em necrose, hemorragia e infiltrado inflamatório neutrofílico e linfocítico. A ampla distribuição de *Merremia macrocalyx* na região estudada e a boa palatabilidade associada à alta sensibilidade da espécie bovina verificada neste experimento, reforça a importância desta planta em casos espontâneos de intoxicação em bovinos.

TERMOS DE INDEXAÇÃO: Intoxicação espontânea, intoxicação experimental, *Merremia macrocalyx*, Convolvulaceae, bovinos, Zona da Mata, Pernambuco, plantas tóxicas, intoxicação por plantas, timpanismo, ruminantes, toxicoses.

INTRODUCTION

Merremia genus encompasses a range of 80 species occurring throughout tropical and subtropical regions (Austin 1982). In Brazil 14 of these species can be found in different environments and locations such as forest borders, countrysides and wastelands (Austin 1982, Simão-Bianchini et al. 2016). *Merremia macrocalyx* belongs to the Convolvulaceae family, it is a herbaceous climbing plant capable of flowering and growing fruits all year round, mainly in the summer, and can be found in the Americas, extending from the United States to Argentina (O'Donell 1941). Considered an invasive plant (Lorenzi 1982, Kissmann & Groth 1992, Lorenzi & Souza 1999), *M. macrocalyx* has already been found in all Brazilian states (Simão-Bianchini et al. 2016, Ferreira & Miotto 2013).

Although several species of the Convolvulaceae family are nontoxic and important fodder plants widely used for animal feeding (Cook et al. 2013), many toxic plants belonging to this family, especially those in the Ipomoea genus, have been reported causing disease and significant economic losses to livestock production in the Brazilian semiarid region (Mendonça et al. 2011, 2012, Oliveira et al. 2011, Tokarnia et al. 2012, Lima et al. 2013, Oliveira et al. 2013). There are no reports of poisoning by *Merremia* species in ruminants in Brazil. However, some species are considered toxic in the United States, Oceania, India and Trinidad and Tobago, such as *Merremia tuberosa* (Ghosh & Ghosh 2010), *M. peltata* (Williams 2012) and *M. dissecata* (Williams & Williams 1969, Mansur 2001). *Merremia tuberosa* has hallucinogenic effects in humans, *M. dissecata* is toxic to ruminants due to its alkaloids and glycosides content, and *M. peltata* has been reported as toxic to fish (Williams & Williams 1969, Mansur 2001, Ghosh & Ghosh 2010, Williams 2012).

The aim of the this study was to describe the epidemiological, clinical and pathological aspects of spontaneous poisoning by *Merremia macrocalyx* in cattle in the Forest Zone of Pernambuco and replicate experimentally the toxicity of this plant in cattle.

MATERIALS AND METHODS

The epidemiological study was carried out in the Forest Zone of Pernambuco, northeastern Brazil, in the municipalities of Condado, Camutanga, Itaquitinga, Nazaré da Mata, Paudalho and Vicência. Five farms from each municipality were visited in order to investigate the occurrence of spontaneous poisonings by *Merremia macrocalyx*.

All information concerning distribution, period of occurrence and clinical signs caused by *M. macrocalyx* consumption in cattle were obtained through a questionnaire based on Silva et al. (2006). Subsequently, pasture areas were surveyed to verify the presence of *M. macrocalyx* and the farms that presented areas containing the plant were monitored for 24 months.

Merremia macrocalyx leaves were collected in Condado and sent to the Poisonous Plant Laboratory, Agricultural Research Service, at the United States Department of Agriculture, Logan/UT, USA for bromatological analysis. For botanical identification, samples were sent to the "Instituto Agronômico de Pernambuco" (IPA). In addition, *M. macrocalyx* samples collected in Condado and Vicência were tested for the presence of hydrocyanic acid (Tokarnia et al. 2000) and nitrates (Riet-Alvariza 1993). Colorimetric method for nitrate percentage determination was also applied (Embrapa 1997).

In order to carry out the experiment with cattle, the precepts related to animal welfare and ethics were followed, as recommended by the National Council for the Control of Animal Experimentation (CONCEA) and the Ethics Committee on the Use of Animals of the Federal Rural University of Pernambuco (CEUA-UFRPE).

For the experimental poisoning, six crossbred Girolando steers, with age ranging from 12 to 24 months were used. The animals were clinically healthy and weighing between 106 and 195kg. Thirty days before the beginning of the experiments, cattle received antiparasitic medication and were kept in a shared facility, aiming adaptation and previous clinical observation. During the adaptation period, every day, early in the morning, steers received commercial ration, mineral salt, Napier grass (*Pennisetum purpureum*) minced in the trough and water *ad libitum*. After that, animals were kept in paddocks and then taken back to the shared facility at dusk. Prior to the beginning of the experimental trial, cattle were fasted for 12 hours and then distributed into three groups: Group I (control group) was composed by two steers under the same management conditions as the others, however these animals did not consume *Merremia macrocalyx*. Group II, consisting of two cattle that received fresh leaves of *M. macrocalyx* in the trough at a single dose of 60g/kg, and Group III, composed by two cattle that received fresh leaves of *M. macrocalyx* in the trough at a single dose of 80g/kg.

The animals were clinically examined throughout the experiment period, twice daily. Clinical exams were performed according to Dirksen et al. (1993); hemogram, urinalysis, ruminal fluid analysis (Kaneko et al. 2008) and a test to verify diphenylamine content were also performed. Blood collection was carried out on the first, third and fifth day of the experiment, and a diphenylamine test was performed after the first clinical signs were detected. For physical and chemical evaluation of ruminal fluid, the pH of samples was checked immediately using pH indicator strips. Afterwards, color, odor, appearance, flotation-sedimentation, methylene blue reduction and protozoan activity were evaluated (Dirksen 1993). Three evaluations of ruminal fluid were performed. The first evaluation

was performed prior to the administration of *Merremia macrocalyx* leaves, second evaluation 24 hours after administration, and the third was performed on the 8th day of experiment on those that survived the poisoning.

Cattle that died were submitted for necropsy, and samples from organs of the abdominal and thoracic cavity, as well as the encephalon were collected, fixed in 10% formalin, embedded in paraffin, cut into sections ranging from 5-6µm and stained by hematoxylin-eosin (HE) for histological examination. The experimental design is shown in Table 1.

RESULTS

Spontaneous poisoning by *Merremia macrocalyx*

Considering the applied questionnaire, poisoning episodes occurred in farms which shared similar features among each other, such as areas of poorly managed pastures of up to 10 hectares. Outbreaks occurred in periods of low rainfall from August to March. In Condado, *Merremia macrocalyx* (Fig.1) was five times mentioned as responsible for the deaths of 35 cattle and two poisoning outbreaks were followed. No outbreak was reported in Camutanga and Itaquitinga and in visited sites the plant was not found. In Nazaré da Mata, the plant was mentioned twice as responsible for the death of 27 sheep and three cattle. In Vicência a poisoning outbreak was observed, and in Paudalho it was mentioned twice. In these places *M. macrocalyx* was found in pasture areas and on the banks of the side roads.

Poisoning outbreaks in Condado occurred in November 2013 and February 2014. The first cases were identified during the removal of a batch of five cattle grazing in an area invaded by *Merremia macrocalyx*. Three out of the five animals consuming the plant presented clinical signs that consisted of restlessness,

Table 1. Experimental poisoning by *Merremia macrocalyx* in cattle, general data

Cattle			Daily dose (g/Kg)	Daily dose consumed (Kg)	Clinical signs onset	Clinical signs intensity*	Evolution	Outcome (days)
Group	Number	Weight (Kg)						
GI	1	165	Control	-	Absent	Absent	-	-
	2	178	Control	-	Absent	Absent	-	-
GIII	3	180	60	10.8	9h	+++	Recovered	7
	4	195	60	11.7	8h	++	Recovered	8
GIII	5	106	80	8.48	12h	+++	Died	3
	6	175	80	14	14h	+++	Died	2

* + Mild, ++ moderate, +++ severe.

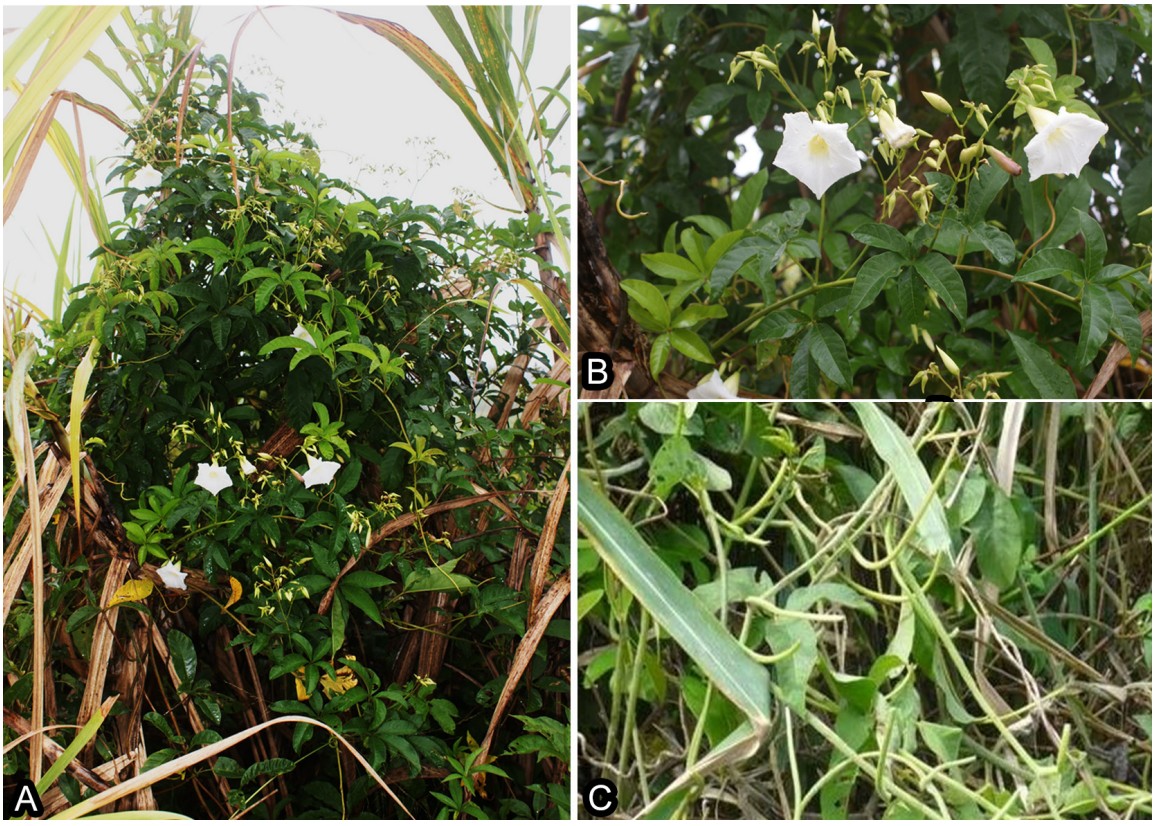


Fig.1. (**A**) *Merremia macrocalyx* in cattle grazing area. The plant is observed covering other shrubs, Condado, Pernambuco. (**B**) Leaves and flowers. (**C**) *Merremia macrocalyx* showing evidence of cattle consumption.

tympanism, ruminal atony, regurgitation of ruminal fluid, polyuria, diarrhea and death 48 to 72 hours after the onset of clinical signs (Fig.2A). In the referred farm, two cattle died, one of which was necropsied. The third one recovered completely within 15 days after ruminal transfaunation. In this farm, deaths in cattle in the previous years with a similar clinical condition were reported, usually including tympanism. In the second outbreak, out of a total of 19 cattle, five showed mild to moderate clinical signs, mainly tympanism and on that occasion one of the animals died and was necropsied.

The third outbreak was observed in Vicência in December 2015. Out of a total of 16 cattle, two presented tympanism, prolonged recumbency, ruminal atony and death in less than 24 hours. The pastures of the three properties were composed of *Brachiaria decumbens* and in all of them *Merremia macrocalyx* specimens were observed with signs of being consumed by cattle.

Necropsy findings in two spontaneously poisoned cattle were similar and consisted of tympanism (Fig.2B), blood vessels congestion and petechiae in the rumen serosa. Rumen, omasum and reticulum contents were dry and compacted and abomasum content was liquid (Fig.2C). Abomasal mucosa was hyperemic, with swollen folds and contained multiple ulcers with a maximum of 0.5cm in diameter. Cattle had epicardial petechiae and lung consolidation areas and edema in the pulmonary caudal lobes. Histologically, the main lesions consisted of acantholysis, ballooning degeneration of rumen and reticulum keratinocytes, sometimes with the formation of pustules and inflammatory infiltration of polymorphonuclear cells and lymphocytes in the epithelium and the submucosa had marked edema (Fig.2D-F). The abomasum mucosa showed multifocal areas of necrotic epithelium, severe neutrophilic and lymphocytic inflammatory infiltrate; edema, congestion and hemorrhage in the submucosa were also noted. Similar

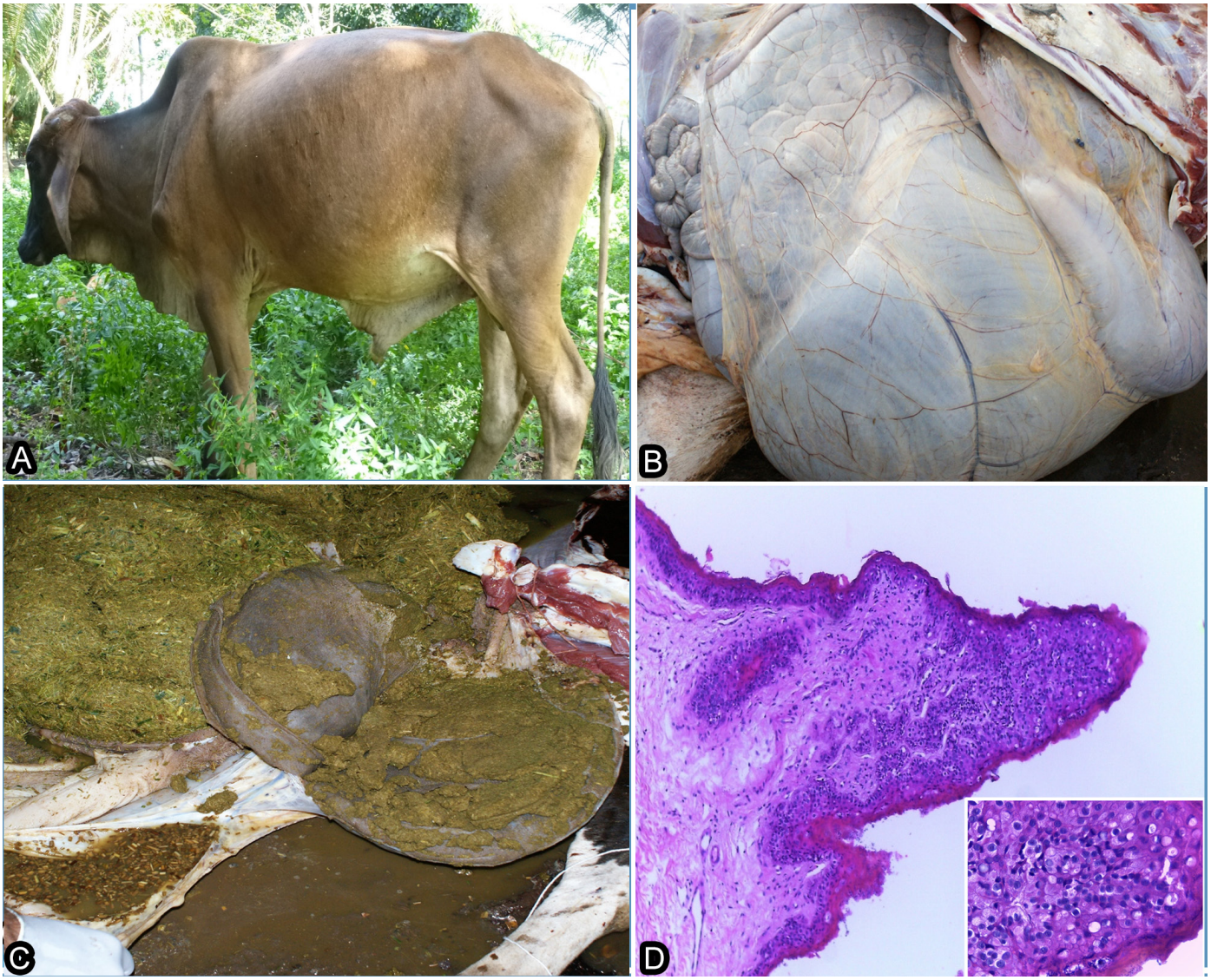


Fig.2. (**A**) Spontaneously poisoned cattle with severe tympanism. (**B**) Rumen, reticulum and abomasum show enlargement due to gas accumulation. (**C**) Dry and impacted rumen and reticulum contents and excessively liquid abomasum content. (**D**) Rumen mucosa presenting keratinocytes and epithelial necrosis with ballooning degeneration, lymphocytic and neutrophilic inflammatory infiltrate containing bacteria and abscesses. HE, obj.10x.

lesions, characterized by necrosis and inflammation of the lining epithelium, edema and hemorrhage of the submucosa were also present in the duodenum.

Bromatological analysis of *Merremia macrocalyx* leaves demonstrated crude protein (29%), digestibility (89%) and neutral detergent fiber (27%). Picro-sodium paper test had a negative reaction, diphenylamine test had a weak blue reaction for nitrates and samples of *M. macrocalyx* collected from Condado and Vicência indicated 0.3% nitrate concentrations in dry matter.

Experimental poisoning by *Merremia macrocalyx* in cattle

Animals that ingested *Merremia macrocalyx* developed clinical signs within 8 to 14 hours after total consumption and the clinical outcome of the disease lasted from 2 up to 8 days (Table 1).

Poisoning clinical signs varied in severity degree yet all animals presented apathy, anorexia, restlessness, irritability, ocular discharges, sialorrhea, dehydration, polyuria and diarrhea. In addition to these clinical signs, individuals that consumed 60g/kg of *Merremia macrocalyx* leaves presented ruminal motility reduction, mild to moderate tympanism, and fully recovered within 7-8 days. Those in the 80g/kg daily dose presented ruminal motility reduction followed by atony, tympanism, dyspnea, orthopneic posture, muscle tremors, weakness, sternal recumbency and death within 2 or 3 days (Fig.3A). There was a decrease in methylene blue reduction activity and ruminal fluid pH was alkaline in all cattle, reaching values between 7.5-8.0. Density and motility of protozoa declined progressively only in the most severe cases of intoxication; the percentage of mortality ranged from 60% to 80%. No significant changes were observed

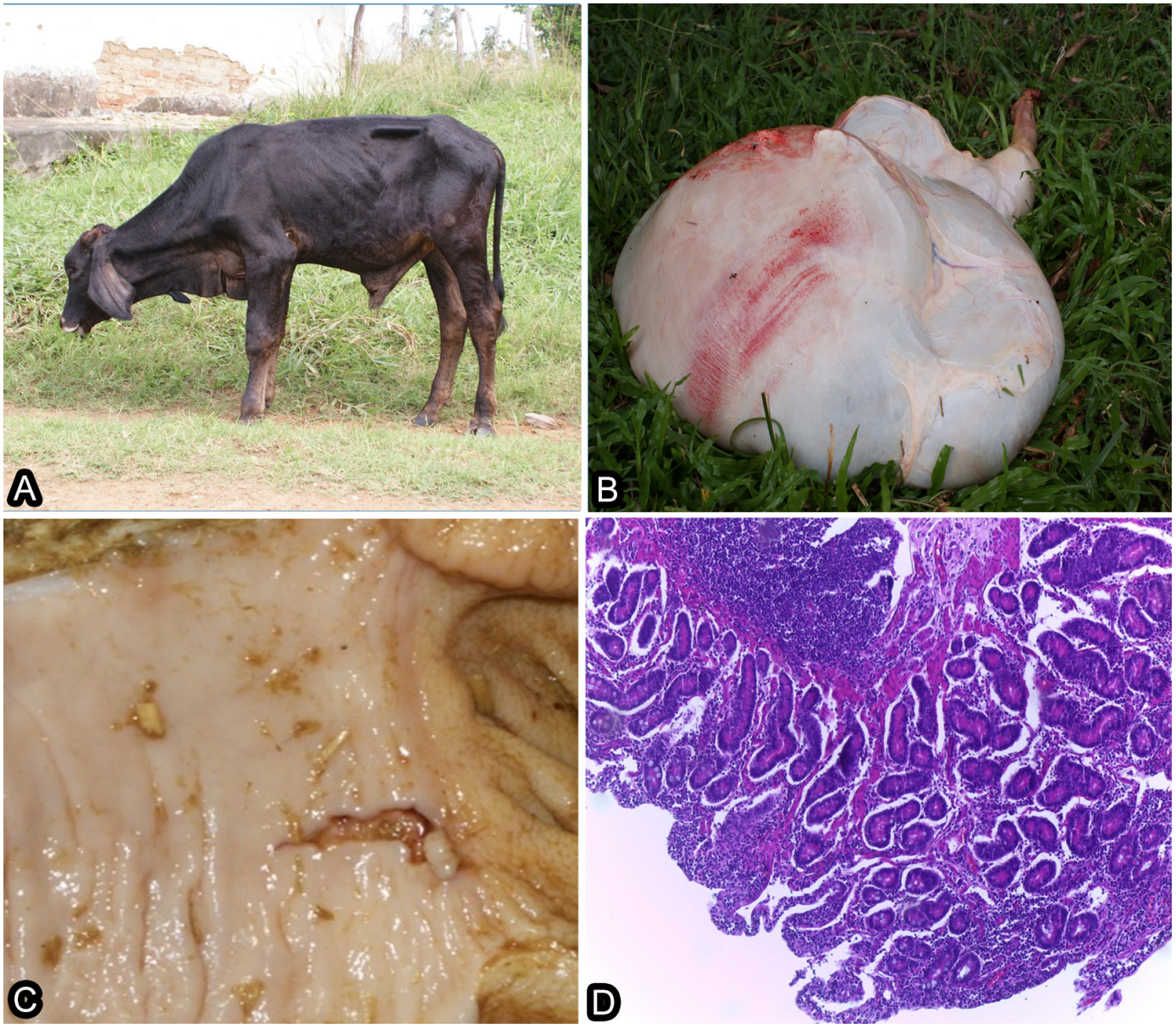


Fig.3. (**A**) Experimentally poisoned cattle presenting apathy and orthopneic posture due to digestive system lesions. (**B**) Multifocal areas of hemorrhage are observed in the rumen serosa. (**C**) Duodenum mucosae presenting erosive lesions. (**D**) Epithelial necrosis, severe neutrophilic and lymphocytic inflammatory infiltrate of the duodenal mucosae. HE, obj.10x.

in experimentally poisoned cattle erythrograms. However, there was discreet leukocytosis due to neutrophilia and lymphocytosis. There were no significant changes in the biochemical parameters analyzed.

The group fed with 80g/kg *Merremia macrocalyx* leaves presented similar lesions to those spontaneously poisoned; these lesions consisted of rumen and reticulum distention with dry contents and petechial hemorrhages in rumen serosa (Fig.3B). Abomasum contents were liquid, there was edema of folds and ulcers with petechial hemorrhages in the mucosa; ulcers were also observed in duodenal mucosa (Fig.3C). Histological findings consisted of rumen and reticulum epithelial necrosis with neutrophils and lymphocytes inflammatory infiltrate, in addition to edema and hemorrhage. Intestinal villi epithelial necrosis with lymphocytic and polymorphonuclear cells inflammatory infiltrate and congestion of the submucosa vessels were also observed in the small intestine, mainly in the duodenum (Fig.3D-F).

DISCUSSION

Considering the outbreaks of *Merremia macrocalyx* poisoning and its wide distribution in the Forest Zone of Pernambuco, we can say that this is a toxic plant species of interest for livestock that causes acute tympanism. Currently, due to fodder scarcity, ranchers kept cattle confined or tied by ropes in *M. macrocalyx* higher concentrations areas for the cattle to consume it as source of fodder in drought periods when this plant is found abundantly and remains green for long periods of time. Therefore, main factors favoring cattle poisoning were food shortages, good plant palatability and its presence in poorly managed pasture areas.

Farmers' reports suggest that sheep are also poisoned spontaneously, however, in this species, acute bloat outbreaks have not been identified and need to be investigated. In addition, due to the *Merremia macrocalyx* occurrence throughout the Brazilian national territory (Simão-Bianchini et al. 2016, Ferreira & Miotto 2013), veterinarians from other states should be vigilant and should include *M. macrocalyx* poisoning in the differential diagnosis when outbreaks of tympanism are observed in cattle.

Poisoning in cattle had subacute to acute clinical progression, with gas bloating representing the most characteristic clinical sign of the disease; main lesions consisted of necrosis and inflammation of the mucosa of the rumen, reticulum, abomasum and duodenum. The most likely hypothesis for this poisoning pathogenesis is related to the plant toxins action that may cause disturbances in the nerve pathways involved in eructation reflex, since rumen and reticulum mucosa (which contain mechanoreceptors and voltage receptors that discriminate gas, foam and liquid) injuries may disrupt normal reflex which is essential for rumen gas removal (Forbes 1986, Allen & Mertens 1988, Allen 1996, Radostitis et al. 2002, Andrews et al. 2008).

Merremia macrocalyx toxins responsible for the lesions in the digestive tract of cattle are unknown. However, alkaloids, phenolic compounds and glycolipids are the biologically active constituents most commonly found in various species of Convolvulaceae extracts (Meira et al. 2012). In a previous study with leaves and roots of *M. macrocalyx*, saponins, quinones, steroidal triterpenes, polyphenols and tannins were detected (Bakasso et al. 2008). In other *Merremia* species, such as *M. tuberosa*, *M. peltata*, *M. dissecta* and *M. guerichii* toxic alkaloids were also found (Nahrstedt et al. 1989, Jenett-Siems et al. 2005, Austin 2007). In another study, 18 distinct types of pyrrolizidine alkaloids were identified in different *Merremia* species (Jenett-Siems et al. 2005). For this reason, the content of alkaloids in *M. macrocalyx* leaves should be investigated, since it is known that these nitrogen compounds have as main function to avoid herbivory and, therefore, may be related to the pathogenesis of such intoxication in cattle (Perez et al. 2015).

In Brazil, despite numerous toxic plants of economic importance, there are few with action on the digestive system of cattle (Tokarnia et al. 2012). There is only one species of plant, *Trifolium pratense*, which causes acute tympanism in cattle in southern Brazil. In these cases, however, tympanism is foamy and occurs due to excessive fermentation of soluble carbohydrates and proteins of high ruminal degradability in plants (Gava 1990, Dalto et al. 2009). The most important species that affect the digestive system are Baccharis coridifolia in the South and *Stryphnodendron coriaceum* in the Northeast of Brazil (Rissi et al. 2005, Mello et al. 2010). The clinical-pathological signs induced by these plants in cattle are similar to that of *M. macrocalyx* poisoning and include lesions characterized by necrosis and inflammation of the digestive system; without significant alterations in other organs. There is no treatment for *M. macrocalyx* poisoning in cattle, but in spontaneous cases, clinical improvement was observed after oro-ruminal probe and support therapy to correct hydroelectrolytic disorders.

CONCLUSION

Merremia macrocalyx is a relevant toxic plant for ruminants that causes tympanism and lesions in the digestive system of cattle in the Forest Zone of Pernambuco state, Brazil.

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Ethics committee.- The experiment was approved by the animal ethics committee of UFRPE (Authorization number 100/2015).

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

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Original Article
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Subgingival bacterial microbiota associated with ovine periodontitis[1]

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ABSTRACT.- Silva N.S., Borsanelli A.C., Gaetti-Jardim Júnior E., Schweitzer C.M., Silveira J.A.S., Bomjardim H.A., Dutra I.S. & Barbosa J.D. 2019. **Subgingival bacterial microbiota associated with ovine periodontitis.** *Pesquisa Veterinária Brasileira 39(7):454-459*. Laboratório de Patologia Clínica Veterinária, Faculdade de Medicina Veterinária, Instituto de Medicina Veterinária, Universidade Federal do Pará, Campus de Castanhal, Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mail: nataliasilvasilveira1@gmail.com

Periodontitis is an inflammatory response in a susceptible host caused by complex microbiota, predominantly composed of Gram-negative anaerobic bacteria. Aiming to characterize the subgingival bacterial microbiota associated with ovine periodontitis, polymerase chain reaction (PCR) was performed in subgingival periodontal pocket samples of 14 sheep with severe periodontitis and in subgingival sulcus biofilm of 14 periodontally healthy sheep in search mainly of Gram-negative and Gram-positive microorganisms considered important periodontopathogens. The most prevalent bacteria in the sheep with periodontal lesions were *Tannerella forsythia* (78.6%), *Treponema denticola* (78.6%), *Fusobacterium nucleatum* (64.3%), and *Porphyromonas gingivalis* (50%), whereas in the healthy sheep, *F. nucleatum* (42.8%) was the most often detected bacterium. Statistically significant differences were observed for *Campylobacter rectus, Enterococcus faecium, Prevotella nigrescens, T. forsythia*, and *T. denticola* ($p<0.05$) in the sheep with periodontitis in the comparison between groups. *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis,* and *Porphyromonas gulae* were not detected in any of the samples analyzed. In conclusion, *C. rectus, E. faecium, P. nigrescens, T. forsythia,* and *T. denticola* were associated with severe lesions caused by ovine periodontitis, and *F. nucleatum* was the most prevalent microorganism in the subgingival sulcus biofilm of healthy sheep.

INDEX TERMS: Subgingival, bacteria, microbiota, periodontitis, periodontal disease, anaerobic microorganisms, sheep, polymerase chain reaction, bacterioses.

RESUMO.- [**Microbiota bacteriana subgengival associada à periodontite ovina.**] Periodontite é a resposta inflamatória de um hospedeiro suscetível causada por complexa microbiota, composta predominantemente por bactérias anaeróbias Gram-negativas. Com o objetivo de caracterizar a microbiota bacteriana subgengival associada à periodontite ovina foi realizada a reação em cadeia da polimerase (PCR) de amostras de biofilme subgengival de 14 ovinos com a enfermidade e 14 ovinos periodontalmente saudáveis, com destaque para micro-organismos Gram-negativos e Gram-positivos considerados importantes periodontopatógenos. As bactérias mais prevalentes em 14 animais com lesões periodontais foram *Tannerella forsythia* (78,6%), *Treponema denticola* (78,6%), *Fusobacterium nucleatum* (64,3%) e *Porphyromonas gingivalis* (50%). Entretanto, nos 14 ovinos sem lesões periodontais, *F. nucleatum* (42,8%) foi a bactéria mais detectada. Associação estatisticamente diferente foi observada para *Campylobacter rectus, Enterococcus faecium, Prevotella nigrescens, T. forsythia* e *T. denticola* ($p<0,05$) nos ovinos

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com periodontite em comparação entre os dois grupos. *Aggregatibacter actinomycetemcomitans, Enterococcus faecalis* e *Porphyromonas gulae* não foram detectados em nenhuma das amostras pesquisadas. Conclui-se que *C. rectus, E. faecium, P. nigrescens, T. forsythia* e *T. denticola* estão associados às lesões resultantes da periodontite ovina com manifestação clínica grave e *F. nucleatum* o micro-organismo mais prevalente no biofilme subgengival de animais periodontalmente sadios.

TERMOS DE INDEXAÇÃO: Microbiota, bactéria, subgengival, periodontite, ovinos, doenças periodontais, micro-organismos anaeróbios, reação em cadeia da polimerase, bacterioses.

INTRODUCTION

Periodontium is the set of tissues that support and protect the teeth comprising the gingiva, periodontal ligament, root cementum, and alveolar bone proper. Periodontitis is a multifactorial infectious disease caused by complex microbiota that interacts with the tissues and cells of a susceptible host, causing the release of a broad array of inflammatory mediators that lead to destruction of the structures that surround and support the dental elements (Holt & Ebersole 2005, Feng & Weinberg 2006).

Among several periodontopathogens, bacteria of Socransky Red Complex, namely, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola*, have been considered the main microorganisms causing periodontitis in humans and animals because of their virulence factors and strong association with the disease (Ashimoto et al. 1996, Mayanagi et al. 2004, Feng & Weinberg 2006). However, recent studies involving metagenomics and metatranscriptomics suggest that the development of periodontitis is associated with more diverse microbiota (Dewhirst et al. 2010, Griffen et al. 2012, Jorth et al. 2014). Thus, the disease results from polymicrobial synergy and dysbiosis, which promote imbalance of the periodontal biofilm associated with homeostasis (Hajishengallis 2015). Dysbiosis is characterized by an imbalance in the relative abundance of species within a microbial community compared with its abundance in health, leading to a change in the host-microorganism ratio sufficient to trigger destructive inflammatory response (Hajishengallis 2014).

In animals raised extensively, high prevalence of periodontitis was described in calves in Brazil in the 1960s, initially associated with the rearing of cattle on newly formed pastures in former native forest and *cerrado* areas. At the time, it was considered the most important disease in cattle breeding, and limited livestock development in the Southeast and Center-West regions (Döbereiner et al. 1974, 2000). Outbreaks with high prevalence and severity also occurred in pastures replanted in previously endemic areas, which led to serious economic losses to the livestock sector (Dutra et al. 1993, 1999). In conventional microbiological analysis of lesions in bovine periodontitis using traditional methods of anaerobic culture, characterization and identification, it is possible to observe that approximately 80% of the microbiota of calves associated with periodontitis was composed of black pigmented colonizing bacteria when cultured in blood agar supplemented with hemin and vitamin K (Botteon et al. 1993, Dutra et al. 1999). With the use of molecular techniques such as polymerase chain reaction (PCR), which presents high sensitivity and specificity, several species of the genera *Porphyromonas*, *Prevotella* and *Treponema* (Borsanelli et al. 2015a, 2015b) were identified in bovine periodontitis, considered as potential pathogens in humans, dogs, cats, sheep, and goats.

Sheep present a clinical condition of periodontitis that may cause premature loss of incisor teeth (Spence et al. 1988). Studies have reported that, in this animal species, the oral microbiota of periodontitis is compatible with that found in human periodontitis (Frisken et al. 1989, Ismaiel et al. 1989, McCourtie et al. 1989, 1990, Duncan et al. 2003). Riggio et al. (2013) evaluated the presence of specific bacterial species in sheep samples with and without periodontitis and emphasized that there were clear differences in the composition of the microbiota between ill and healthy animals. Specimens of two genera of periodontopathogens, *Porphyromonas* and *Prevotella*, belonging to the Socransky Red and Orange Complexes, showed close association with lesions of ovine periodontitis (Borsanelli et al. 2017). Likewise, some species of spirochetes of genus *Treponema* have also been identified in sheep with periodontitis (Borsanelli et al. 2016).

The use of traditional culture methods for detection of oral microorganisms presents operational difficulties, from limitations of biochemical and physiological methods in the precise identification of pathogens to challenges in maintaining the nutritional requirements and oxygen sensitivity of isolates, interfering with the performance of field studies (Ashimoto et al. 1996). In addition, it is estimated that only 3% of bacteria can be cultured *in vitro*; 97% of bacteria cannot be cultured (Davis 2016). In this sense, detection through amplification of the target DNA by PCR is adequate, because it is a highly sensitive and specific technique that enables direct identification of bacteria in biological samples and detection of uncultured microorganisms or of those that are difficult to characterize. Therefore, molecular methods can be used in the detection and evaluation of possible associations between specific microorganisms and periodontitis (Ashimoto et al. 1996).

Silva et al. (2016) reported an outbreak of periodontitis in a sheep herd in the state of Pará, Brazil, with high prevalence and severe clinical signs of periodontitis, weight loss, death, and epidemiological conditions similar to those occurring in episodes of bovine periodontitis. Therefore, the objective of the present study was to identify, through PCR, the presence of specific periodontopathogens in samples of periodontal pocket in animals with periodontitis and of subgingival sulcus biofilm in periodontally healthy sheep.

MATERIALS AND METHODS

Clinical characterization of periodontitis and sample collection. The study sample was composed of Santa Inês, Dorper and Texel sheep and their crossbreeds aged 5 months to 5 years. Their clinical status was established after extra-and intra-oral examination including periodontal evaluation, verification of attachment loss, tooth mobility, and purulent exudate in the maxillary or mandibular premolar and molar teeth.

Animals were raised under confinement and semi-confinement systems. They were fed *Panicum maximum* cv. Massai and elephant grass (*Pennisetum purpureum*) and received mineral mixture and brewery waste supplemented with sodium chloride solution *ad libitum*.

Samples of purulent exudate of the periodontal pocket of 14 sheep with severe clinical signs of periodontitis were obtained during an outbreak of the disease, reported by Silva et al. (2016), in a property located in the municipality of Benevides, state of Pará, Brazil. Samples

were collected only from periodontal pockets with depth greater than 5mm using sterile swabs, transferred to cryotubes containing ultrapure water, and stored in liquid nitrogen until processing.

Sampling of subgingival sulcus biofilm from periodontally healthy animals was performed in a different herd. The periodontal clinical condition of the animals was classified as healthy when the surrounding gingiva was firm, without bleeding at probing, and when gingival sulcus depth did not exceed 4mm. Thus, samples of the subgingival biofilm were collected from 14 Sulfolk sheep considered periodontally healthy by the paper cone test according to the procedures described by Gaetti-Jardim et al. (2012). The sheep were raised under an extensive grazing system on *Panicum maximum* cv. Massai pasture. After the outbreak, this stage of the study was approved by the Ethics Committee on Animal Experimentation (FOA process no. 2015-00280). The paper cones were transferred to cryotubes containing 1ml sterile ultrapure water and stored at -80°C until DNA extraction.

Bacterial identification by polymerase chain reaction (PCR). DNA extraction was performed in the material collected from the periodontal pocket and subgingival sulcus biofilm using a commercial kit (GenElute Mammalian Genomic DNA Miniprep Kit, Sigma). The search of *Aggregatibacter actinomycetemcomitans*, *Campylobacter rectus*, *Eikenella corrodens*, *Enterococcus faecalis*, *Enterococcus faecium*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Porphyromonas gulae*, *Prevotella intermedia*, *Prevotella nisgrescens*, *Tannerella forsythia* and *Treponema denticola* was conducted with the use of specific primers (Ashimoto et al. 1996, Gaetti-Jardim et al. 2010, Kato et al. 2011) (Table 1). The specificity of these primers has been demonstrated in the aforementioned literature and can be evaluated at the National Center for Biotechnology Information (NCBI) databases.

Target DNA PCR amplification was performed in 25µL volumes containing 1X PCR/Mg+2 buffer (Boehring Mannheim, Indianapolis/IN, USA), 0.2µl of each dNTP (Pharmacia Biotech, Piscataway/NJ, USA), 0.5U Taq DNA polymerase (Invitrogen do Brasil, São Paulo/SP, Brazil), 0.4µL of each primer pair (Invitrogen), and 10ng model. Amplification was performed in thermocycler (Perkin Elmer, GeneAmp PCR System 9700, Norwalk/CT, USA) programmed at 94°C (5min), 30 to 40 cycles at 94°C (30 to 60s, according to the primers used), specific annealing temperature for each pair of primers, 72°C (30 to 60s), followed by a final extension step of 5min at 72°C. PCR amplification products were analyzed by 1% agarose gel electrophoresis performed in TBE buffer (1M Tris, 0.9M boric acid, 0.01M EDTA, pH 8.4), stained with ethidium bromide (0.5mg/mL), and visualized under ultraviolet light (UV Light Transilluminator - Eastman Kodak Co., NY, USA).

As control of the detection procedures, DNA samples from standard strains of the studied microorganisms and clinical samples positive for the target microorganisms were used (Gaetti-Jardim et al. 2010, 2012); ultrapure water was used as negative control.

Statistical analysis. Data were processed and analyzed using the SPSS software. Associations between the groups of animals with and without periodontitis and the bacterial species were verified by the Student's *t*-test and the Spearman's correlation test. Statistical tests underwent the Bonferroni correction with *p* value adjusted from 0.05 to 0.000003 because 10 microbial species were detected.

RESULTS

The most prevalent microorganisms in the 14 sheep with severe periodontitis were *Tannerella forsythia* (78.6%), *Treponema denticola* (78.6%), *Fusobacterium nucleatum* (64.3%) and *Porphyromonas gingivalis* (50%), whereas in the gingival sulcus of the 14 periodontally healthy sheep, *F. nucleatum* (42.8%) was the most often detected microorganism. *Aggregatibacter actinomycetemcomitans*, *Enterococcus faecalis*, and *Porphyromonas gulae* were not detected in any of the samples analyzed (Table 2).

Table 1. Primers used in the polymerase chain reaction (PCR) to identify the bacteria of Socransky Red Complex and other microorganisms present in the subgingival microbiota of sheep with periodontitis

Species	Primers (5'-3')	Annealing temperature	Amplicon (bp)
Aggregatibacter actinomycetemcomitans	AAA-CCC-ATC-TCT-GAG-TTC-TTC-TTC ATG-CCA-ACT-TGA-AGT-TAA-AT	55	557
Campylobacter rectus	TTT-CGG-AGC-GTA-AAC-TCC-TTTT-C TTT-CTG-CAA-GCA-GAC-ACT-CTT	60	598
Eikenella corrodens	CTA-ATA-CCG-CAT-ACG-TCC-TAA-G CTA-CTA-AGC-AAT-CAA-GTT-GCC-C	60	688
Fusobacterium nucleatum	ATT-GTG-GCT-AAA-AAT-TAT-AGT-T ACC-CTC-ACT-TTG-AGG-ATT-ATA-G	40	1010
Porphyromonas gingivalis	AGG-CAG-CTT-GCC-ATA-CTG-CG CTG-TTA-GCA-ACT-ACC-GAT-GT	60	404
Porphyromonas gulae	TTG-CTT-GGT-TGC-ATG-ATC-GGG-CT TAT-TCT-TAC-GGT-ACA-TTC-ACA	60	314
Prevotella intermedia	CGT-GGA-CCA-AAG-ATT-CAT-CGG-T CTT-TAC-TCC-CCA-ACA-AAA-GCA	55	575
Prevotella nigrescens	ATG-AAA-CAA-AGG-TTT-TCC-GGT-AAG CCC-ACG-TCT-CTG-TGG-GCT-GCG-A	55	804
Tannerella forsythia	GCG-TAT-GTA-ACC-TGC-CCG-CA TGC-TTC-AGT-GTC-AGT-TAT-ACC-T	60	641
Treponema denticola	TAA-TAC-CGA-ATG-TGC-TCA-TTT-ACA-T TCA-AAG-AAG-CAT-TCC-CTC-TTC-TTC-TTA	60	316
Enterococcus faecalis	ATC-AAG-TAC-AGT-TAG-TCT ACG-ATT-CAA-AGC-TAA-CTG	45	941
Enterococcus faecium	TTG-AGG-CAG-ACC-AGA-TTG-ACG TAT-GAC-AGC-GAC-TCC-GAT-TCC	65	658

Table 2. Periodontal pathogens detected in the periodontal pocket of sheep with periodontitis (n=14) and in the subgingival sulcus biofilm of periodontally healthy animals (n=14) by PCR

Species	Periodontal pocket n (%)	Subgingival sulcus biofilm n (%)	*p*
Aggregatibacter actinomycetemcomitans	0 (0.0)	0 (0.0)	
Campylobacter rectus	6 (42.9)	0 (0.0)	0.0043*
Eikenella corrodens	4 (28.6)	1 (7.1)	0.1493
Enterococcus faecalis	0 (0.0)	0 (0.0)	
Enterococcus faecium	6 (42.9)	0 (0.0)	0.0043*
Fusobacterium nucleatum	9 (64.3)	6 (42.9)	0.2722
Porphyromonas gingivalis	7 (50.0)	3 (21.4)	0.1233
Porphyromonas gulae	0 (0.0)	0 (0.0)	
Prevotella intermedia	3 (21.4)	0 (0.0)	0.0709
Prevotella loescheii	1 (7.1)	0 (0.0)	0.3265
Prevotella nigrescens	6 (42.9)	0 (0.0)	0.0043*
Tannerella forsythia	11 (78.6)	0 (0.0)	0.0000*
Treponema denticola	11 (78.6)	0 (0.0)	0.0000*

* Values are statistically significant when $p<0.05$ by the Student's *t*-test.

Statistical analysis showed positive correlation between *Campylobacter rectus*, *Enterococcus faecium, Prevotella nigrescens, Tannerella forsythia*, and *Treponema denticola* and ovine periodontitis (Table 2).

DISCUSSION

Dental changes are considered one of the main reasons for the early slaughter of sheep, within their normal breeding period, thus increasing the costs of animal replacement (West 2002). In this context, periodontal disease and excessive tooth wear are the main disturbances affecting the tooth supporting structures and teeth of sheep (West & Spence 2000). In Brazil, ovine periodontitis has been diagnosed in the North (Silva et al. 2016) and Southeast (Borsanelli et al. 2016, 2017, Agostinho 2017) regions. In the studied herds, this disease was responsible for serious economic losses due to animal weight loss and death (Silva et al. 2016).

Periodontal infection in sheep resulted from an acute process, with severe gingival retraction, attachment loss of the premolar and molar teeth, purulent exudate, bulging of the mandible or maxilla, teeth loss, defects in occlusion, difficulty in rumination, emaciation, and eventual death of animals, clinically characterizing a severe condition (Silva et al. 2016). Despite the growing evidence of primary infectious etiology in periodontal diseases in human medicine and in some animal species, few studies have highlighted the role of specific subgingival microbiota in periodontal disease in ruminants. In the present study, it was possible to associate the occurrence of *Tannerella forsythia, Treponema denticola, Prevotella nigrescens, Enterococcus faecium*, and *Campylobacter rectus* with ovine periodontitis.

In ruminants, *Tannerella forsythia* has been identified in sheep (McCourtie et al. 1989, Duncan et al. 2003, Agostinho 2017), cattle (Borsanelli 2017), and goats (Campello 2017) with periodontitis. This microorganism has been frequently detected in human patients with periodontitis (Cortelli et al. 2005, Foschi et al. 2005, Feng & Weinberg 2006, Yoo et al. 2007, Gaetti-Jardim et al. 2010, Benrachadi et al. 2012) and, initially, it was believed to be a relatively uncommon species found in the subgingival sulcus biofilm. Presently, it is known that its levels are strongly associated with increased depth of the periodontal pocket. This species is observed in larger number, proportion and prevalence in individuals with periodontitis than in healthy individuals. In this context, this bacterium can also be detected in a larger number of sites with destructive periodontal disease than in healthy sites. In addition, it was detected in larger numbers in progressive active periodontal lesions compared with inactive lesions (Socransky & Haffajee 2010).

Inclusion of *Treponema denticola* in the group of microorganisms studied was also based on the evidence that spirochetes play an important role in the etiology of human and animal periodontitis. Among its peculiarities, there is a high number of sites with increased pocket depth and location at the margin of periodontal lesions, which facilitates its adherence to epithelial cells and the invasion of adjacent tissues that should be highlited. Healthy sites show few or none spirochetes and sites with gingival inflammation but without attachment loss, exhibit low to moderate indexes, whereas deep periodontal pockets harbor a large number of these bacteria (Socransky & Haffajee 2010). *T. denticola* is considered one of the major periodontopathogens in humans (Mayanagi et al. 2004, Foschi et al. 2005, Feng & Weinberg 2006, Gaetti-Jardim et al. 2010, Benrachadi et al. 2012), and it has also been identified in periodontal lesions in bovines, sheep and goats (Borsanelli et al. 2015a, 2016, Agostinho 2017, Borsanelli 2017, Campello 2017).

Prevotella nigrescens and *Prevotella intermedia* cannot be distinguished by conventional culture methods (Ashimoto et al. 1996). In the present study, it was possible to verify the presence of both species in the periodontal lesions of sheep, but only *P. nigrescens* showed significant association with the disease. *P. nigrescens* has been identified in bovines and sheep with periodontitis (Borsanelli et al. 2015a, 2017), and is frequently detected in the subgingival sulcus biofilm of healthy human patients with periodontitis (Mayanagi et al. 2004).

In human periodontitis, *Campylobacter rectus* is present in larger numbers in diseased sites (Doğan et al. 2003) with active periodontal destruction (Rams et al. 1993) and in individuals with severe periodontitis (Gajardo et al. 2005). In livestock, *C. rectus* has been identified in sheep, goats and

cattle with periodontitis (Agostinho 2017, Borsanelli 2017, Campello 2017). This microorganism has also been identified in dogs with periodontitis (Nishiyama et al. 2007, Hirai et al. 2013) and in robust capuchin monkeys with gingivitis and periodontitis (Gaetti-Jardim et al. 2012).

In humans, detection of enteric bacteria in periodontal pockets seems to be inversely proportional to the presence of anaerobic periodontopathogens (Botero et al. 2007); however, the opposite was observed in the present study, and *Enterococcus faecium* showed association with ovine periodontal lesions.

Considering the limited number of primers used in the present study, other microorganisms were surely present in the subgingival microbiota of sheep with periodontitis. However, the presence of periodontally pathogenic species, such as *Tannerella forsythia*, *Treponema denticola* and *Prevotella nigrescens*, brings important elements to elucidate the polymicrobial and multifactorial etiology of ovine periodontitis and understand the consequences of this infectious process.

Direct examination of material from the periodontal lesions of the sheep through PCR, with its recognized sensitivity and specificity, enabled detection of microorganisms of complex culture, such as *T. denticola*, and avoided the use of laborious and not always successful steps in the isolation of non-sporulated anaerobic microorganisms (Socransky & Haffajee 2010). In the same context, it was possible to observe absence of *Enterococcus faecalis* in ovine microbiota, as well as absence of two of the most important human periodontopathogens, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gulae*, associated with periodontitis in dogs (Dahlén et al. 2012).

The taxonomic changes that have occurred in several periodontopathogens and the current methods for detecting bacteria in the subgingival microbiota hinder the accurate establishment of parallels between the findings of the present study and the species isolated from the periodontal pocket in calves with "cara inchada" (Blobel et al. 1984, Dutra et al. 1986, Botteon et al. 1993). Although a preliminary comparative analysis of ovine periodontitis can establish points in common with microbiological studies, the clinical-pathological and epidemiological situation of this outbreak of periodontitis in sheep in addition to the presence of periodontopathogens resemble that occurred in bovine periodontitis (Dutra et al. 1999, Döbereiner et al. 2000). It should be emphasized that the present study describes the occurrence of microorganisms with periodontal pathogenic potential, using a qualitative molecular technique that met the purposes of the study and added objective complementary information on the possible causes of ovine periodontitis. Thus, this is an important step in elucidating the causal factors associated with the disease, which, as a multifactorial, infectious, polymicrobial disease associated with complex dental biofilm, should fill the Socransky's postulate, and not the classic Koch's postulates.

CONCLUSIONS

Campylobacter rectus, Enterococcus faecium, Prevotella nigrescens, Tannerella forsythia and *Treponema denticola* are associated with lesions resulting from ovine periodontitis with severe clinical manifestation.

Fusobacterium nucleatum was the most prevalent microorganism in the subgingival sulcus biofilm of periodontally healthy sheep.

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Original Article
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Epidemiological study of rabies in cattle in southern Brazil: spatial and temporal distribution from 2008 to 2017[1]

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ABSTRACT.- Santos B.L., Bruhn F.R.P., Coelho A.C.B., Estima-Silva P., Echenique J.Z., Sallis E.S.V. & Schild A.L. 2019. **Epidemiological study of rabies in cattle in southern Brazil: spatial and temporal distribution from 2008 to 2017**. *Pesquisa Veterinária Brasileira 39(7):460-468*. Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário s/n, Pelotas, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

Rabies affects several domestic species, causing significant economic losses due to the death of animals in characteristic areas of the southern region of Rio Grande do Sul. In this context, this work aimed to study the epidemiology of rabies outbreaks observed in cattle from January 2008 to December 2017, through its space and time distribution in the southern region of Rio Grande do Sul. We performed an observational and ecological retrospective through the analysis of data recorded in necropsy protocols performed in cattle at the "Laboratório Regional de Diagnóstico" of the "Universidade Federal de Pelotas" (LRD-UFPel). A descriptive data analysis aimed at evaluating the distribution of cases over time. The statistical analysis was performed with Gretl statistical software 1.9.12 (GNU Regression, Econometric, and Time-series Library). To the existence of stationarity was verified with the Dickey-Fuller test, considering a value of $p<0.05$. From January 2008 to December 2017, 1418 bovine diagnostic materials were received at the LRD-UFPel, and 160 outbreaks of rabies were confirmed in 160 farms located in the 24 municipalities of the LRD-UFPel area of influence. We observed 591 cattle out of a total of 25,886 infected with the virus. We conclude that rabies does not exhibit seasonality in the study region, with a tendency to decrease in frequency in the next years. The disease has an epidemic characteristic in most of the analyzed months (2012-2014), remaining endemic in the remaining period (2011, 2015 and 2016) with sporadic episodes of epidemics in these years. We also observed that as the incidence of the disease increased in the animals and in the properties, there was a geographical spread of the virus for the majority (54.16%) of the municipalities in the area of influence of the LRD-UFPel.

INDEX TERMS: Epidemiology, rabies, cattle, southern Brazil, descriptive analysis, mortality, epidemics.

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RESUMO.- [Estudo epidemiológico da raiva em bovinos no Sul do Brasil: distribuição espacial e temporal de 2008 a 2017.] A raiva afeta várias espécies domésticas, causando perdas econômicas significativas pela morte de animais em áreas bem características da região Sul do Rio Grande do Sul. Neste contexto, o objetivo deste trabalho foi estudar a epidemiologia dos surtos de raiva observados em bovinos de janeiro de 2008 a dezembro de 2017, por meio de sua distribuição no espaço e no tempo na região sul do Rio Grande

do Sul. Foi realizado um estudo observacional e ecológico retrospectivo, por meio da análise de dados registrados nos protocolos de necropsias realizadas em bovinos no Laboratório Regional de Diagnóstico da Universidade Federal de Pelotas (LRD-UFPel). Foi feita análise descritiva dos dados para avaliar a distribuição dos casos ao longo do tempo. Para a análise temporal foi utilizado o software estatístico Gretl 1.9.12 (GNU Regression, Econometricand Time-series Library). Para verificar a existência da estacionariedade utilizou-se o teste de Dickey-Fuller aumentado, considerando um valor de p<0,05. No período de janeiro de 2008 a dezembro de 2017, foram recebidos no LRD-UFPel 1418 materiais de bovinos para diagnóstico, sendo confirmados 160 surtos de raiva em 160 propriedades situadas nos 24 municípios da área de influência do LRD-UFPel. O número de animais infectados pelo vírus foi de 591 bovinos de um total de 25.886. O presente estudo permitiu concluir que a raiva não tem sazonalidade na região do estudo, tendo tendência de queda na frequência nos próximos anos. A doença tem característica epidêmica na maioria dos meses analisados (2012-2014), mantendo-se endêmica no período restante (2011, 2015 e 2016) com episódios esporádicos de epidemias nestes anos. Observou-se ainda, que na medida em que aumentou a incidência da doença nos animais e nas propriedades, houve disseminação geográfica do vírus para a maioria (54,16%) dos municípios da área de influência do LRD-UFPel.

TERMOS DE INDEXAÇÃO: Epidemiologia, raiva, bovinos, Sul do Brasil, análise descritiva, séries temporais, mortalidade, epidemia.

INTRODUCTION

Rabies is a neurological condition caused by a virus of the genus *Lyssavirus*. Affecting humans and almost all domestic and wild mammals that act as reservoirs of the disease, including the hematophagous bat *Desmodus rotundus,* the main transmitter of the disease to cattle, its evolution is invariably fatal. The disease is endemic in many parts of the world and a significant zoonosis with global mortality estimated around 40,000-100,000 humans per year (Fernandes & Riet-Correa 2007).

In South America, rabies in herbivorous populations appear in cyclical outbreaks, occurring approximately every seven years (Hudson et al. 1996). The enzootic stability of rabies in the state of Rio Grande do Sul is due to favorable climatic conditions, the presence of natural and artificial shelter for the bat, which allows the rabies virus to subsist in nature, and the distribution of bovine populations throughout the entire territory. Although canine rabies has been successfully controlled in the state, the bovine condition is still endemic in some regions. The National Program for Rabies Control in Herbivores (Programa Nacional de Controle da Raiva dos Herbívoros - PNCRH) was created in Brazil with the aim of reducing the disease prevalence in the domestic herbivore population (Brasil 2017).

According to data published by the Regional Diagnosis Laboratory at "Universidade Federal de Pelotas" (LRD-UFPel) from 1978 to 2015, 6,228 samples from cattle were received; among them, 206 were diagnosed with rabies, 3.3% of the total of bovine diagnostics in the laboratory (Schild et al. 2017). The 24 municipalities covered by LRD-UFPel are located in six of the 34 micro-regions of Rio Grande do Sul, which holds approximately 20% of the state's cattle with an effective livestock of approximately 2.8 million animals (Vergara 2017), which demonstrates the importance of this study in Rio Grande do Sul.

Research on the spatial and time distribution of rabies conducted in the states of Minas Gerais, São Paulo, and Bahia shows the influence of factors such as climate, temperature, and rainfall on the occurrence of outbreaks of rabies in certain areas (municipalities), seasons (seasonality), and throughout the years (cyclicity) (Silva et al. 2001, Menezes et al. 2008, Gomes et al. 2011, Teixeira et al. 2015).

In this context, this work aimed to study the epidemiology of outbreaks of rabies observed in cattle from January 2008 to December 2017 by means of their time and spatial distribution in the state of Rio Grande do Sul, as well as to identify the distribution of multifold outbreaks of the disease in the region after successful control for a period of 12 years.

MATERIALS AND METHODS

A retrospective study, carried out by collecting information on the occurrence of rabies in cattle in different municipalities of the state of Rio Grande do Sul from 2008 to 2017, by means of the analysis of data from records of bovine necropsies performed at LRD-UFPel.

The study region encompassed the LRD-UFPel area of influence (southern Rio Grande do Sul), consisting of 24 municipalities. These municipalities belong to six out of the 35 micro-regions of the state: 1 - the micro-region of Pelotas (municipalities of Arroio do Padre, Canguçu, Capão do Leão, Cerrito Alegre, Cristal, Morro Redondo, Pedro Osório, Pelotas, São Lourenço do Sul, and Turuçu); 2 - Campanha Meridional (Aceguá, Bagé, Dom Pedrito, Hulha Negra e Lavras do Sul); 3 - Campanha Central (São Gabriel e Santana do Livramento); 4 - Serras do Sudeste (Candiota, Encruzilhada do Sul, Pedras Altas, Pinheiro Machado, Caçapava do Sul, Piratini e Santana da Boa Vista); 5 - Jaguarão (Arroio Grande, Herval e Jaguarão); and 6 - Litoral Lagunar (Rio Grande e Santa Vitória do Palmar) (Fig.1).

Data processing and analysis. We collected epidemiological data of each outbreak or isolated case diagnosed at LRD/UFPel. The animals' municipality of origin was recorded along with the month and year when the disease occurred. Additionally, information

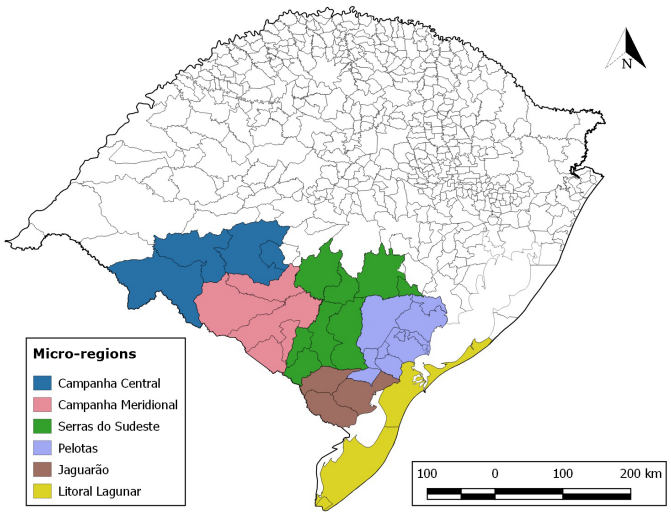


Fig.1. Micro-regions of the LRD-UFPel area of influence (southern Rio Grande do Sul).

on the number of estates and infected animals, as well as the location of each estate as per the Global Positioning System (GPS) was also obtained.

Data were analyzed by means of spatio-temporal statistical methods. For such, the following indicators related to the disease morbidity were built: i - animal incidence rate (AIR) = positive animals/total of animals raised in the municipalities studied (x1000000); ii - incidence rate per estate (EIR) = positive estates/total of estates within the area covered by LRD/UFPel (x100000); iii - incidence rate among estates experiencing outbreaks (OEIR) = positive estates/estates experiencing outbreaks (x100); iv - incidence rate per municipality (MIR) = positive municipalities/total of municipalities within the area covered by LRD-UFPel (x100); v - rabies-specific mortality rate (RMR) = positive animals/animals dying from any bovine disease within the area covered by LRD-UFPel (x100). From this data, a bivariate descriptive statistical analysis was carried out using as dependent variable the incidence of rabies in the animals, the incidence in the estates, and in the municipalities experiencing cases of the disease, and as independent variable, the year, month, micro-region, and municipality.

Time series. Data were analyzed by means of an assessment of case distribution over time and a verification of changes in disease behavior over the years, especially with regard to morbidity and the sensibility of the system employed by the LRD-UFPel. The statistical software Gretl 1.9.12 (GNU Regression, Econometric and Time-series Library) was used for the temporal analyses. An analysis of the existence of trend and seasonality components was carried out by means of verification of the series and their correlograms. Following verification of the components, the series were differentiated and then statistically tested as to the existence of stationarity by means of augmented Dickey-Fuller tests (Dickey & Fuller 1979), considering a value of $p<0.05$ (Baptista et al. 2016, Morettin & Toloi 2006). When stationarity was observed in the differenced series, the presence or absence of the trend and seasonality components in the monthly series considered was inferred. By calculating Ordinary Least Squares (OLS) models, we also estimated the statistically significant trend ($p<0.05$) and calculated the coefficient of determination (R^2) in the time series of indicators over time.

With a view to characterizing the dynamics of occurrence of endemics and epidemics related to cattle morbidity by rabies in southern Rio Grande do Sul (RS), control diagrams were built based on the case distribution from 2011 to 2016 according to the methodology of geometric mean of the rates, more appropriate when an asymmetrical distribution of cases over the years is observed, according to Bortman (1999). The original data has been logarithmically transformed (Log10). The geometric means and confidence intervals at 95% (CI 95%) were calculated from the transformed values. The CIs were used as the lower endemic and epidemic thresholds and the endemic curve (Bortman 1999).

Spatial analysis. The distribution of rabies cases across all of the municipalities was analyzed from thematic maps built with software ArcGis 10.2.1 for desktop. We used Shapefiles, the format of spatial data storage consisting in the position, shape, and attributes of geographical characteristics provided by IBGE (2018). The maps were projected per year based on overlapping cases of rabies in each area of the municipality.

We used the hot spot analysis tool to identify spatial groups from the identification of high (hot spots) and low values (cold spots) (Anselin 1995) of rabies incidence in cattle. According to Getis & Ord (1992), the hot spot analysis calculates the concentration or the lack of concentration of the sum of values associated with the variables in a given data set. Assuming a normal distribution standard, when the probability associated to a z score is positively or negatively higher than a specific level of significance (p value), one obtains a positive or negative spatial association. Therefore, for positive z scores, the intensity of hot spot spatial grouping increases when the z score is higher. On the other hand, for negative z scores, the intensity of cold spot spatial grouping increases when the z score is lower.

RESULTS

From January 2008 to December 2017, the LRD-UFPel received 1418 samples from cattle for diagnosis, including bodies, heads or encephalons, and other organs. During this period, 160 outbreaks of rabies were identified. They took place in 160 estates located in the 24 municipalities within the area of influence of the LRD-UFPel. A number of 591 cattle were infected with the virus out of a total of 25,886 animals raised in the estates infected.

From 2008 to 2011, the rabies virus affected cattle in only four out of the 24 municipalities, all of which belonging to the micro-region of Pelotas. However, from 2012 to 2014, the disease was diagnosed in 13 out of the 24 municipalities (54.16%) belonging to the micro-regions of Pelotas, Serra do Sudeste, Campanha Meridional, and Jaguarão. In 2015, micro-region Campanha Central also recorded cases of the disease. That year, seven municipalities were affected by outbreaks of rabies; in 2016, the number increased to eight and, in 2017, it went down to three.

The highest rabies incidence rate in cattle took place in Capão do Leão, state of RS, with 3.6 cases of infection/1,000,000 animals (Table 1). The highest incidence rate among the estates located in the 24 municipalities within the LRD-UFPel area of influence also took place in Capão do Leão, i.e. six out of 100 thousand cattle farms were infected with the rabies virus (Table 1).

The distribution of rabies in cattle shows 24 outbreaks from 2008 to 2011, which represents 15% of the outbreaks observed in the study period. From 2012 to 2014, 110 outbreaks were diagnosed, which represents 68.75% of the total. From 2015 to 2017, 26 outbreaks occurred, 16.25% of the total. The annual incidence rates of rabies cases observed in the LRD-UFPel area of influence are presented in Table 2.

According to the distribution of rabies over the year, we observe that the highest incidence rate on the animals and estates occurred in June, considering the total of estates in the 24 municipalities. The highest incidence rate among estates experiencing rabies outbreaks occurred from February to June. The highest incidence rate among the municipalities included in the study was observed in March. With regard to the rabies-specific mortality rate within the LRD-UFPel area of influence, the highest percentage was observed between March and April (Table 3).

The statistical analysis of the time series from 2013 to 2016 showed absence of seasonality ($p>0.05$) and presence of trend ($p<0.05$) for all of the indicators analyzed (Fig.2 and 3).

The endemic curves of the animal incidence rate and the incidence rate among estates in the study area from 2011 to 2016 are presented in Figure 4.

The descriptive spatial analysis of the cases of rabies in cattle showed dissemination of the disease across the LRD-UFPel area of influence from 2011 to 2014. In 2011, cases were limited to the micro-region of Pelotas; in 2012,

Table 1. Incidence rate of rabies in the 24 municipalities within the LRD area of influence, Rio Grande do Sul, from 2011 to 2017

Micro-regions	Municipalities	AIR[a]	EIR[b]	OEIR[c]	RMR[d]
Pelotas	Arroio do Padre	0.10	0.30	0.62	23.08
	Canguçu	1.93	4.82	10.62	22.86
	Capão do Leão	3.60	6.21	13.75	19.96
	Cerrito Alegre	0.32	0.62	1.25	14.81
	Cristal	0.10	0.62	1.25	3.28
	Morro redondo	0.64	3.12	6.87	35.85
	Pedro Osório	0.31	0.84	1.87	2.69
	Pelotas	2.54	8.40	18.75	16.40
	São Lourenço do Sul	2.41	5.00	11.25	16.40
	Turuçu	0.40	1.41	3.12	7.43
Campanha Meridional	Bagé	1.26	0.82	1.87	26.14
	Dom Pedrito	0.22	0.32	0.62	1.87
	Hulha negra	0.11	0.34	0.62	1.41
	Lavras do Sul	1.24	0.81	1.87	26.23
Campanha Central	São Gabriel	0.20	0.30	0.62	100.00
Serras do Sudeste	Candiota	0.03	0.30	0.62	6.67
	Encruzilhada do Sul	0.52	0.30	0.62	75.00
	Pedras Altas	0.31	0.60	1.25	10.11
	Pinheiro Machado	1.24	1.72	3.75	61.54
	Piratini	1.54	2.24	5.00	28.39
	Santana da Boa vista	0.43	1.71	3.75	29.73
Jaguarão	Arroio Grande	1.13	1.71	3.75	21.02
	Herval	1.00	1.42	3.12	30.47
	Jaguarão	0.03	0.30	0.62	0.53

[a] AIR = positive cattle/total of animals raised in the LRD-UFPel area of influence (x100).(x1000000)/10, [b] EIR = positive estates/total of estates in the municipalities included in the study (x100000)/10, [c] OEIR = positive estates/estates presenting outbreaks (x100), [d] RMR = positive animals/animals dying from any disease.

Table 2. Incidence rate of rabies, per year, in the municipalities within the LRD area of influence, Rio Grande do Sul, from 2008 to 2017

Year	AIR[a]	EIR[b]	OEIR[c]	MIR[d]	RMR[e]
2008	4.98	14.02	3.50	8.33	3.79
2009	2.57	11.21	2.78	8.33	1.50
2010	8.10	8.41	2.10	4.17	5.94
2011	13.00	33.64	8.39	16.67	7.38
2012	54.24	134.59	33.57	50.00	35.63
2013	55.45	109.36	27.27	41.67	24.96
2014	31.33	64.49	16.08	54.17	13.72
2015	30.19	30.84	7.69	29.17	19.52
2016	15.09	3.08	8.39	33.33	8.54
2017	2.45	8.41	2.10	12.5	1.55
Total	21.7	41.81	11.19	25.83	12.25

[a] AIR = positive animals/total of animals raised in the LRD-UFPel area of influence (x1,000,000), [b] EIR = positive estates/total of estates in the municipalities included in the study (x100,000), [c] OEIR = positive estates/estates presenting outbreaks (x100), [d] MIR = positive municipalities/total of municipalities in the LRD-UFPel area of influence (x100), [e] RMR = positive animals/animals dying from any disease among the cattle within the LRD-UFPel area of influence (x100).

they spread to the micro-region of Serra do Sudeste and, in 2013 and 2014, to the micro-region of Jaguarão. From 2015, the number municipalities and micro-regions affected dropped. That year, although the disease spread to the southern rural micro-region, the number of cases compared to previous years increased only in the municipalities of São Lourenço and Bagé. In 2016, incidence of the disease increased only in Lavras do Sul and Capão do Leão and, in 2017, only in Turuçu (Fig.5).

The statistical hot spots analysis ($p<0.05$) showed that the cluster of rabies in the micro-region of Pelotas spread to the micro-region of Serra do Sudeste and Campanha Meridional from 2011 to 2014. From 2015 to 2017, the cluster size in these regions reduced (Fig.6).

Table 3. Incidence rate of rabies, per month, in the municipalities within the LRD area of influence, Rio Grande do Sul, from 2008 to 2017

Months	AIR[a]	EIR[b]	OEIR[c]	MIR[d]	RMR[e]
January	1.13	1.96	4.38	20.83	36.67
February	1.85	4.21	9.38	29.17	57.45
March	2.47	4.77	10.63	41.67	75.79
April	2.74	5.33	11.88	33.33	60.15
May	1.78	5.88	13.13	50.00	38.23
June	1.26	4.21	9.38	33.33	29.84
July	1.88	4.48	10.00	33.33	37.41
August	1.27	1.96	4.38	20.83	27.82
September	2.50	3.36	7.50	20.83	56.15
October	0.99	3.08	6.88	33.33	20.57
November	1.85	3.92	8.75	33.33	51.43
December	0.38	1.68	3.75	20.83	12.22
TOTAL	1.45	3.73	8.34	30.90	42.00

[a] AIR = positive animals/total of animals raised in the LRD-UFPel area of influence (x1,000,000), [b] EIR = positive estates/total of estates in the municipalities included in the study (x100,000), [c] OEIR = positive estates/estates presenting outbreaks (x100), [d] MIR = positive municipalities/total of municipalities in the LRD-UFPel area of influence (x100), [e] RMR = positive animals/animals dying from any disease among the cattle within the LRD-UFPel area of influence (x100).

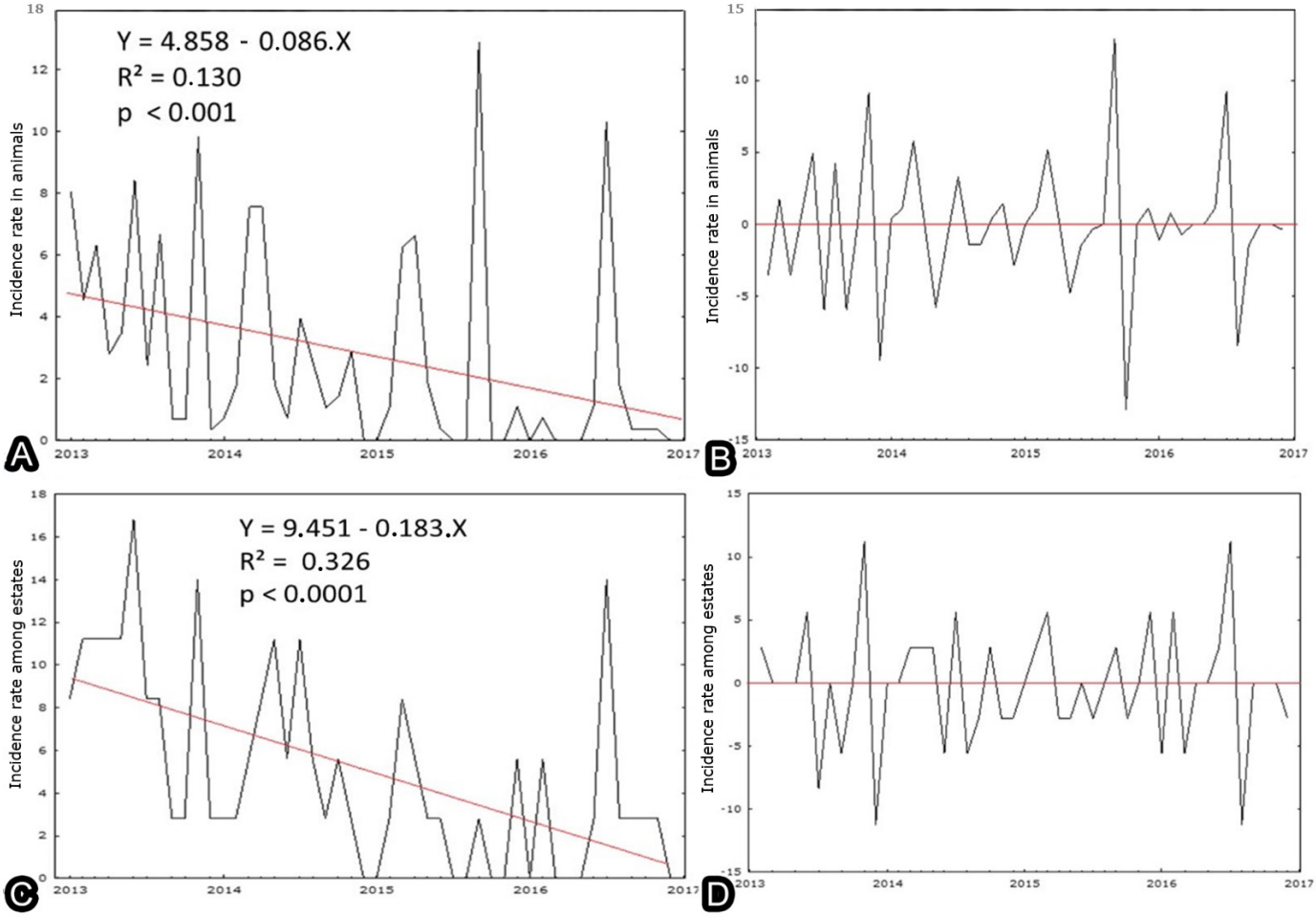


Fig.2. (**A**-**B**) Time series and equation of the animal rabies incidence rate trend line (x 1,000,000 animals) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2013 to 2016. (**A**) Original series and (**B**) trend-free series. (**C**-**D**) Time series and equation of the estate rabies incidence rate trend line (x 100,000 animals) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2013 to 2016. (**C**) Original and (**D**) trend-free series.

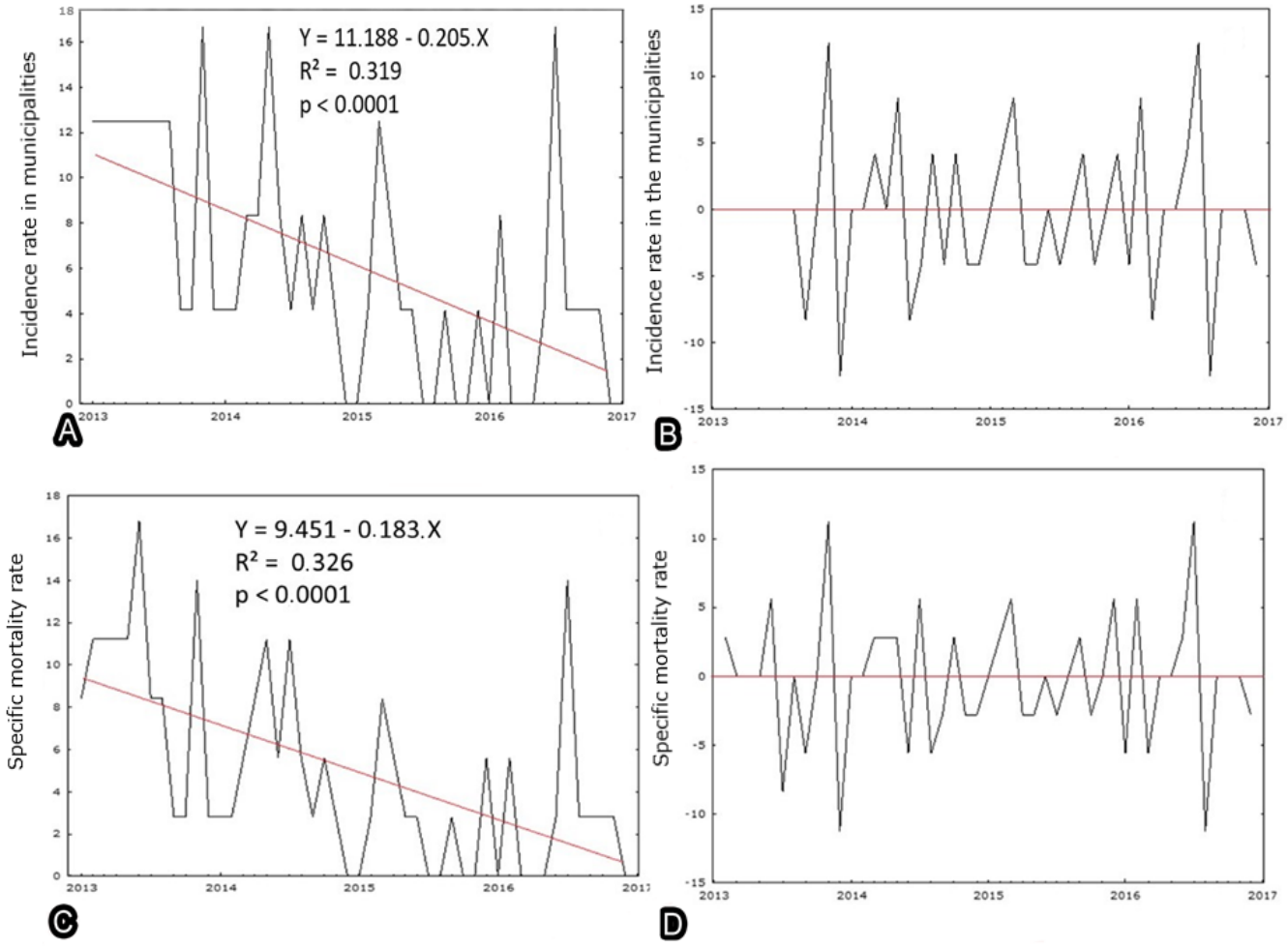


Fig.3. (**A**-**B**) Time series and equation of the municipality rabies incidence rate trend line (1 case per 100 animals) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2013 to 2016. (**A**) Original series and (**B**) trend-free series. (**A**-**B**) Time series and equation of the rabies-specific mortality rate trend line (1 case per 100 animals) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2013 to 2016. (**C**) Original and (**D**) trend-free series.

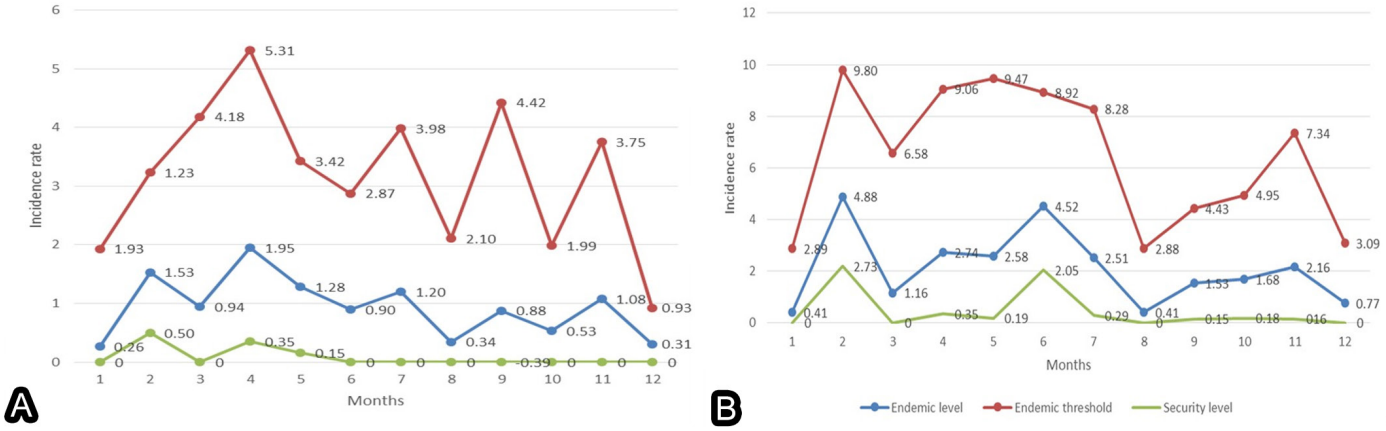


Fig.4. (**A**) Endemic curve of the animal rabies incidence rate (x1000000) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2011 to 2016. x1,000,000 = number of positive animals/total of animals raised in the 24 municipalities. (**B**) Endemic curve of the incidence rate among estates (x100,000) in the LRD-UFPel area of influence, Rio Grande do Sul, from 2011 to 2016. x100,000 = number of positive estates/number of estates in the 24 municipalities.

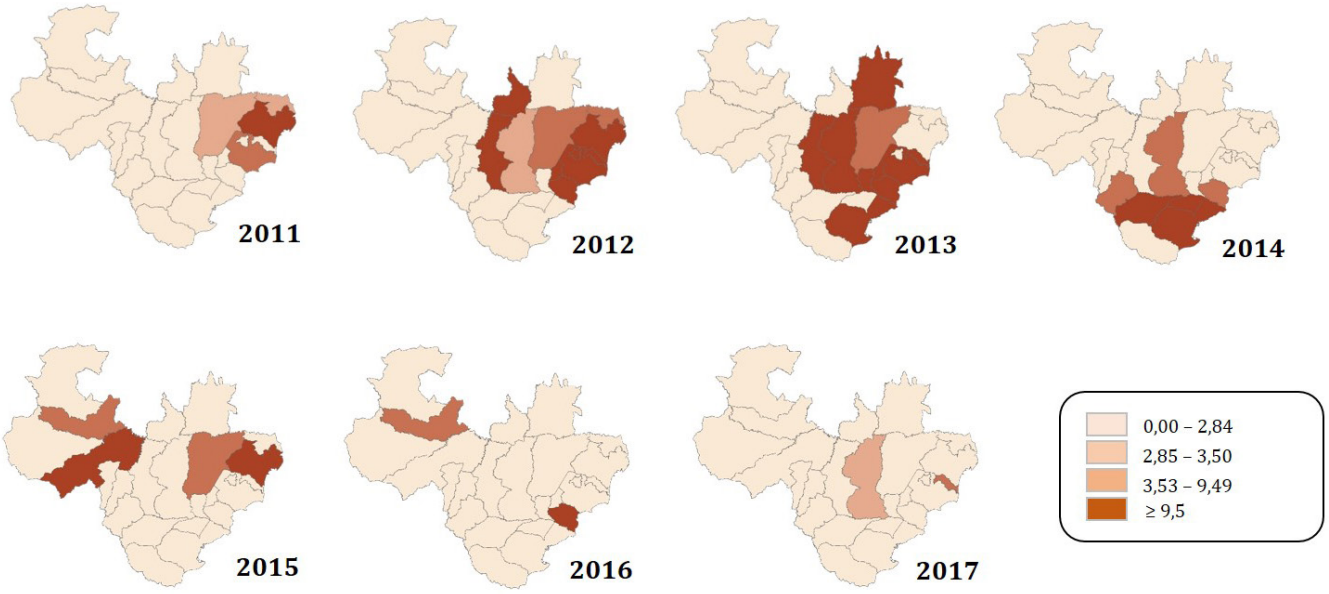


Fig.5. Incidence rate (cases x 1,000,000) of rabies in cattle in the municipalities of the southern region of the state of Rio Grande do Sul from 2011 to 2017.

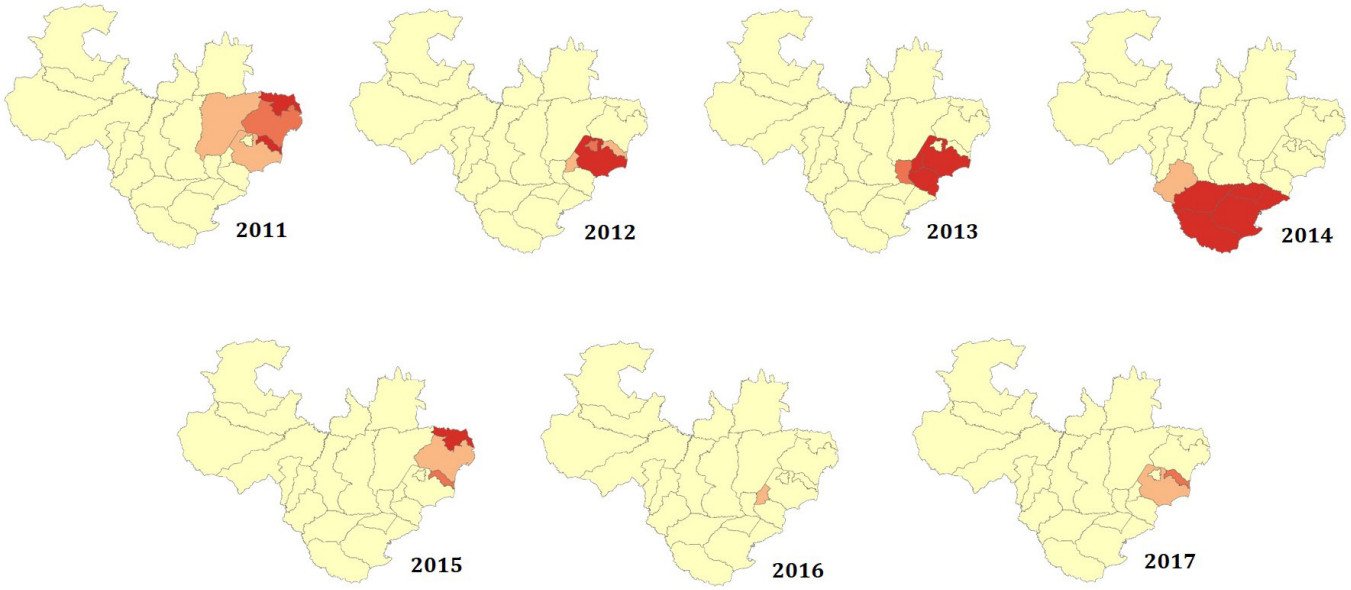


Fig.6. Areas with clusters (hot spot analysis) of rabies cases in cattle in the municipalities of the southern region of the state of Rio Grande do Sul from 2011 to 2017. The hot spot analysis was conducted with software ArcGis 10.2.1. Areas in red indicate clusters with high values (hot spots).

DISCUSSION

The temporal distribution of rabies in cattle surveyed in the 24 municipalities composing the study area showed variation in the number of municipalities with diagnosis of rabies from 2008 to 2017. This data shows that the virus spread to areas without prior record of the disease. This may have been due to a lack of measures against the bat populations that survive under favorable conditions and cross geographical barriers, favoring dissemination of the virus (Fernandes & Riet-Correa 2007, Gomes et al. 2011, Dognani et al. 2016) and a lack of systematic preventive vaccination of the cattle. From 2015, the number of municipalities experiencing outbreaks dropped again, which is probably due to the control measures undertaken in view of the outbreaks diagnosed in the previous years.

Rabies outbreaks occur in areas characteristic of the southern region of the state--on the one hand, the borders of lake Lagoa dos Patos, encompassing areas covered by native vegetation and large centenary fig trees (São Lourenço do Sul, Turuçu, Pelotas, and Cristal); on the other, mountain areas covered by native forest and stone hills with caves (Canguçu, Santana da

Boa Vista, Morro Redondo, Arroio do Padre, Piratini, Pinheiro Machado e Herval). Both regions provide ideal shelters for bat colonies. In addition to shelter, in this region the colonies find food in abundance, especially in the cattle farms (Póvoas et al. 2012). In neighboring municipalities, such as Rio Grande and Arroio Grande, covered by flat, low, and swampy fields, cases of rabies are either inexistent or rare.

The distribution of rabies outbreaks in cattle varied over time. From 2012 to 2014, the incidence rate of the disease in cattle was higher than that found in the other years of the study period. From 2015 to 2017, the number of diagnoses dropped. These results suggest the cyclicity of rabies in this region, although this is not statistically proven. A study conducted in the state of Paraná over a period of 35 years suggests cyclicity of the disease every 18 years (Dognani et al. 2016). On the other hand, studies conducted in the states of Mato Grosso do Sul (Mori & Lemos 1998, Neves 2008, Ribas et al. 2013), São Paulo (Gomes et al. 2011), and Paraíba (Galiza et al. 2010) suggest a period of 5-7 years for cyclicity of rabies in cattle.

The number of cases among animals and in the estates and municipalities is expected to decrease, as a trend of decrease in all of the situations was observed over the study period ($p<0.05$). Menezes et al. (2008) found similar results in a study conducted in Minas Gerais from 1998 to 2006. Another study carried out in Paraná from 1977 to 2012 observed the absence of trend and seasonality (Dognani et al. 2016). Additionally, although some works carried out in the state of RS have suggested that most cases of rabies occur between fall and winter (Marcolongo-Pereira et al. 2011) and spring and summer months (Rech et al. 2006), the disease does not present seasonality in the southern region of RS ($p>0.05$). The absence of seasonality was also observed in studies conducted in the Northeast region of Brazil (Lima et al. 2005) and in the state of São Paulo (Gomes et al. 2011) and Mato Grosso do Sul (Mori & Lemos 1998).

The disease has epidemic characteristics in most of the months analyzed from 2012 to 2014, remaining endemic over the other years (2011, 2015, and 2016) with eventual short periods of epidemics. Other works have shown that rabies is endemic in cattle, presenting variable incidence over the years, with a few epidemic periods throughout the years (Dognani et al. 2016), unlike the findings of this study. In addition, we observed that, as the disease incidence in animals and estates increased, the virus spread geographically to most of the municipalities (54.16%) in the LRD area of influence. Preventive and systematic vaccination of the cattle in these estates is recommended, since the strategy of fighting the bat is not efficient enough to reduce the risks. Furthermore, little is known about the sylvatic cycle of rabies transmitted by wild canids as a source of contamination in addition to bats acting as reservoirs of the virus.

During the study period, we observed not only the dissemination of the disease to unaffected neighboring municipalities, but also the permanence of the disease in previously affected areas. The dissemination of rabies may be related to the geographic expansion of the shelters housing hematophagous bats (Dognani et al. 2016). This study shows that the municipalities presenting cases of rabies have favorable environmental characteristics that provide shelter for the species.

The descriptive spatial and hot spots analyses showed expansion of the disease until 2014 and a period of decrease throughout the following years of the study. Studies on the spatio-temporal distribution of rabies in cattle carried out in the state of Minas Gerais from 1998 to 2016 also found this characteristic (Menezes et al. 2008), as well as a study conducted in the state of Paraná from 1977 to 2012 (Dognani et al. 2016). The decrease in cases of rabies from 2015 may be related to a higher effectiveness of epidemiological surveillance aiming to fight the bat, as well as to the systematic vaccination of the cattle.

CONCLUSIONS

The findings of this study conclude that the incidence of rabies is not associated with seasonality in southern Rio Grande do Sul.

The frequency of cases in the following years seem to tend towards a decrease.

The disease has epidemic characteristics in most of the months analyzed from 2012 to 2014. In the other years (2011, 2015, and 2016), endemic events of epidemics are sporadic.

As the disease incidence in animals and estates increased, the virus spread geographically to most of the municipalities (54.16%) in the LRD area of influence. The spatial analysis showed that the disease spreads mainly due to the geographic proximity between municipalities and neighboring micro-regions.

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

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Equine pythiosis in the eastern wetlands of Uruguay[1]

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ABSTRACT.- Romero A., García J., Balestié S., Malfatto F., Vicentino A., Sallis E.S.V., Schild A.L. & Dutra F.Q. 2019. **Equine pythiosis in the Eastern wetlands of Uruguay.** *Pesquisa Veterinária Brasileira 39(7):469-475*. DILAVE Miguel C Rubino, Laboratorio Regional Este, Avelino Miranda 2045, CP 33000, Treinta y Tres, Uruguay. E-mail: fdutraquintela@gmail.com

Equine pythiosis is an ulcerative and granulomatous disease of the skin, caused by the oomycete *Pythium insidiosum* (Pythiaceae). The objective of this study was to describe seven cases of equine pythiosis that occurred from 2012 to 2017 in the eastern region of Uruguay. Six of the seven cases occurred in the eastern wetland ecosystems of the Merin basin, and the remaining case occurred in the wetland fluvial plains of the Tacuarembó River. Lesions consisted of a large, rapidly growing ulcerated tumor with abundant granulation tissue, serosanguineous secretion, and fistulous tracts containing large concretions or kunkers. The animals presented intense pruritus, claudication and loss of body condition, with death or euthanasia *in extremis* in six cases. The main histological lesions consisted of an eosinophilic and pyogranulomatous inflammatory process, with numerous foci of eosinophilic necrosis (kunkers), collagenolysis, and a Splendore–Hoeppli reaction. In all cases, silver coloration (Grocott) showed intralesional hyphae compatible with *P. insidiosum*, which was confirmed by immunohistochemistry in three cases. A horse in the terminal phase of the disease was treated with triamcinolone acetonide (50mg IM every 15 days), and fully recovered after 1 year. It is concluded that equine pythiosis is prevalent in the wetland ecosystems of eastern Uruguay and that treatment with triamcinolone is auspicious.

INDEX TERMS: Equine, *Pythium insidiosum*, pythiosis, eastern wetland, Uruguay, horses, pathology.

RESUMO.- [**Pitiose equina em áreas úmidas do leste do Uruguai.**] Pitiose é uma doença granulomatosa e ulcerativa da pele dos equinos causada pelo oomyceto *Pythium insidiosum* (Pythiaceae). O objetivo deste trabalho foi descrever sete casos de pitiose equina que ocorreram de 2012 a 2017 na região leste do Uruguai. Seis dos sete casos ocorreram no ecossistema de áreas pantanosas da bacia da bacia da Lagoa Mirim Merin e o restante nas planícies fluviais pantanosas do rio Tacuarembó. As lesões se caracterizaram por tumores ulcerados de crescimento rápido com abundante tecido de granulação, secreção serossanguinolenta e presença de tratos fistulosos contendo material coraloide ou *kunkers*. Os equinos apresentavam prurido intenso, claudicação e perda da condição corporal e seis morreram ou foram eutanasiados *in extremis*. As principais lesões histológicas consistiam de um processo inflamatório piogranulomatoso com numerosos focos de necrose eosinofílicos (*kunkers*), colagenólise e reação de Splendori-Hoepli. Em todos os casos a impregnação pela prata (Grocott) revelou a presença de hifas intralesionais compatíveis com *P. insidiosum*, o que foi confirmado pela imuno-histoquímica em três casos. Um equino em fase terminal da doença foi tratado com triamcinolona acetonida (50mg, IM, a cada 15 dias), recuperando-se completamente após um ano. Conclui-se que a pitiose é uma enfermidade presente em áreas úmidas na região leste do Uruguai e o tratamento com triamcinolona pode ser uma alternativa promissora.

TERMOS DE INDEXAÇÃO: Equinos, *Pythium insidiosum*, pitiose, região úmida, leste uruguaio, Uruguai, patologia.

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INTRODUCTION

Pythiosis, also known as "leeches" in the US, "swamp cancer" in Australia, "bursattee" (i.e. rainy season) in India, "hyphomycosis destruens" in southeast Asia, and "Mal dos Pantanos" in Brazil, is a lethal granulomatous cutaneous disease of horses, caused by the aquatic fungus-like oomycete *Pythium insidiosum* (kingdom Stramenopila, family Pythiaceae) (Santos et al. 2011, Grooters 2013). Pythiosis sporadically occurs in canines, cats, ruminants and humans, although it is not considered to be a disease with zoonotic potential (Gaastra et al. 2010, Mauldin & Peters-Kennedy 2015). The cutaneous/subcutaneous form of pythiosis is the most commonly described form in horses (Chaffin et al. 1995, White et al. 2008, Grooters 2013), although intestinal, nasal and pulmonary cases have also been reported (Goad 1984, Allison & Gillis 1990, Morton et al. 1991, Souto et al. 2016). The cutaneous/subcutaneous form may also involve the regional lymph nodes, or may invade the underlying bone and cause osteomyelitis of the lower limbs (Alfaro & Mendoza 1990). No predilection of race, sex or age exists (Gaastra et al. 2010, Grooters 2013). The disease is found worldwide (especially in Australia, India, Thailand, and southern US), occurring with the highest incidence in tropical and subtropical regions associated with wetlands, dumps or swampy areas (Gaastra et al. 2010). In South America, an extensive series of cases has been reported in Colombia (Cardona-Álvarez et al. 2014), Venezuela (Márquez et al. 2010), and Brazil, particularly in the Pantanal Mato-Grossense (Pereira & Meirelles 2007, Santos et al. 2011) and in lowland areas adjacent to the Merin and Patos lagoons (Marcolongo-Pereira et al. 2012).

Environmental conditions are the most influential factors for the occurrence of equine pythiosis. *Pythium insidiosum* requires a warm-water environment (30-40°C) and an organic substrate of decaying vegetation in order to survive and reproduce (Gaastra et al. 2010). Zoospores show chemotaxis to decomposing plants, as well as to animal hair or damaged tissues. Once in the host, zoospores lose their flagella, encyst, develop germinal tubes, and invade tissues with the help of proteolytic enzymes (Mendoza et al. 1996). The lesions are usually located in the body parts most often in prolonged contact with contaminated water, such as the distal extremities, the ventral aspect of the thoraco-abdominal wall and the face (Grooters 2013, Mauldin & Peters-Kennedy 2015). Lesions begin as small ulcerations that rapidly enlarge to become large tumor-like masses, with multiple fistulous tracts and pale yellow concretions similar to coral, called "kunkers" or "leeches" (Mauldin & Peters-Kennedy 2015). The kunkers, a unique pathological finding of the equine species, exist microscopically in areas of necrosis with large numbers of degranulated eosinophils, collagenolysis, fibrosis, and a Splendore–Hoeppli reaction around P. *insidiosum* hyphae (Miller & Campbell 1984, Martins et al. 2012). Hyphae are difficult to visualize histologically with hematoxylin and eosin (HE) and periodic acid–Schiff (PAS) staining, but are strongly positive to Grocott's methenamine silver (GMS), and can be confirmed with immunohistochemistry (Brown et al. 1988, Martins et al. 2012) or molecular methods (Azevedo et al. 2012).

The treatment of pythiosis is controversial. Surgically removing all affected tissue with a safety margin to avoid recurrences is often hampered in field horses by the size of the lesion, the risky anatomical regions that are typically involved and the high cost of the surgery (Grooters 2013). Surgery followed by immunotherapy is reported to be 70% effective (Pereira & Meirelles 1997, Mendoza & Newton 2005). Antimycotic chemotherapy with potassium iodine or amphotericin B generally fails because *P. insidiosum* is not a fungus and hence does not have ergosterol in its cytoplasmic membranes (Mendoza et al. 1996). Some authors point to the high efficacy of the use of amphotericin B in a 10% solution of dimethylsulfoxide by means of the intravenous distal regional perfusion technique (Dória et al. 2015). An earlier report of several horses cured following the use of the synthetic corticosteroid triamcinolone acetonide intramuscularly (Cuero-Castilla & Mendoza-Espinoza 1985 *apud* Mendoza et al. 1996) was recently confirmed in a case-control study with 100% efficacy in 12 affected horses (Cardona-Álvarez et al. 2016, 2017).

Although Uruguay has favorable ecological conditions for the circulation and transmission of *Pythium insidiosum* (Machado et al. 2018), studies of this disease in Uruguay are scarce and limited to laboratory reports (Dutra et al. 2012, 2014). The objective of the present study was to describe the clinical, pathological and geographical distribution of a series of seven cases of equine pythiosis diagnosed in the eastern region of Uruguay from 2012 to 2017. The successful treatment of a case was also reported.

MATERIALS AND METHODS

A retrospective study of seven cases of pythiosis in horses diagnosed from 2012 to 2017 at the East Regional Laboratory of the DILAVE Miguel C. Rubino, Treinta y Tres, Uruguay, was conducted. Clinical, epidemiological and management data were collected during visits to the farms. Biopsies from the seven horses were fixed in a 4% aqueous solution of buffered formaldehyde, dehydrated in graded alcohol, cleared in xylene, embedded in paraffin, cut into 5µm-thick sections and stained with hematoxylin and eosin (HE), periodic acid–Schiff (PAS), and Grocott´s methenamine silver stain (GMS).

For the confirmation of *Pythium insidiosum* infection, paraffin blocks of all cases with suspicious lesions were immunolabelled using a polyclonal anti-*P. insidiosum* antibody produced in rabbits, using the streptavidin-peroxidase complex and chromogen 3,3 diaminobenzidine (DAB) (Brown et al. 1998). Immunohistochemistry (IHC) was performed at the "Laboratório Regional de Diagnóstico", "Universidade Federal de Pelotas", Pelotas/RS, Brazil.

One horse in the final phase of the disease and with an unfavorable prognosis (Case 7), was treated with triamcinolone acetonide as a last attempt to save the animal, with the agreement of the owner. The dosing protocol recommended by Cardona-Álvarez et al. (2016), 50mg/kg, IM, every 15 days, was followed. The animal was clinically evaluated every 15-20 days for 4 months and then followed-up sporadically until complete remission.

To investigate the geographic pattern of the disease, the seven cases were geolocated on the digital map of the Portal of the National Agricultural Information System (SNIA 2018), using, as geographical coordinates, the rural census track of the paddock where the animal was at the time of diagnosis. A one-to-one point map, with one point representing a single case, was created. In order to reveal the geographic pattern, the following geographic layers were superimposed on the point map: 1) topographic layer; 2) soil types layer (1: 1,000,000); 3) flooding layer (ordinally classified into six growing categories of flooding frequency and duration: occasional, very short, short, irregular long, regular long and very

long); and 4) soil drainage layer (ordinally classified into eight increasing categories of drainage: poor, some poor to poor, some poor, moderate to some poor, moderate, good to moderate, good and excessive) (Molfino & Califra 2004).

RESULTS

Case description

Table 1 shows the clinical and epidemiological data of the seven cases of equine pythiosis diagnosed in the Departments of Treinta y Tres, Durazno and Rocha. All cases occurred from March to June (late summer and autumn). Most of the horses were female (5/7), of the Criollo breed (6/7 cases) and adults (6/7 cases; 5-19 years), although an Arabian mare (Case 2) and a 7-month-old filly (Case 6) were also diagnosed. Two rice-livestock farms had a history of similar cases in previous years.

Clinical signs and pathology

All the affected animals showed rapid deterioration, extensive multifocal alopecia, pain, claudication, and severe pruritus in the affected areas. Lesions were located on the lower lip and chin (Case 1), upper lip and left nostril (Case 2), ventral abdomen (Case 5), distal forelegs (Case 3 and 6), and distal hindlegs (Case 4 and 7). The lesions appeared as large ulcerated masses, 15 to 40cm or more, with an irregular surface and draining tracts that discharged thick strands of a serosanguineous fluid (Fig.1). Yellowish-white concretions, either embedded in the tissue or detached into sinus cavities, were found in all cases (Fig.2). Smaller satellite lesions were observed in horses with large limb lesions.

Histologically, in all horses, the epidermis was extensively ulcerated and covered by dried fibrin, bacteria and cellular debris. In the dermis and subcutaneous panniculus, there was extensive fibroplasia, with multifocal to coalescing areas of eosinophilic necrosis containing karyorrhectic cellular debris, occasional clear hyphal-ghost spaces, and numerous degranulating eosinophils encrusted on degenerating collagen fibers (collagenolysis) (Fig.3). Eosinophils, neutrophils, mast cells and macrophages surrounded these necrotic foci (Fig.4). Occasionally, there were giant cells and foci of Splendore–Hoeppli reaction. With GMS staining, a variable amount of irregular, sparsely septate hyphae with thick, parallel cell walls and branched at 90° angles, was observed (Fig.5). *Pythium insidiosum* was confirmed by IHC in three of the seven cases (Case 2, 5 and 6). PAS staining was inconclusive or negative in all cases.

Evolution and treatment of a case

In six of the seven cases, the lesion extended rapidly over several weeks until death or euthanasia *in extremis*; none had responded to previous antibiotic or antifungal treatment. Horse treated with triamcinolone acetonide (Case 7) had a large lesion in the right hind limb, anemia (11% microhematocrit), and a very poor body condition previous to first dosing (Fig.6A). By 2 weeks after the first dosing, pruritus, bloody secretion and satellite lesions had disappeared, ulceration progressively regressed, the surface became smooth and pink, hematocrit increased to 28%, and

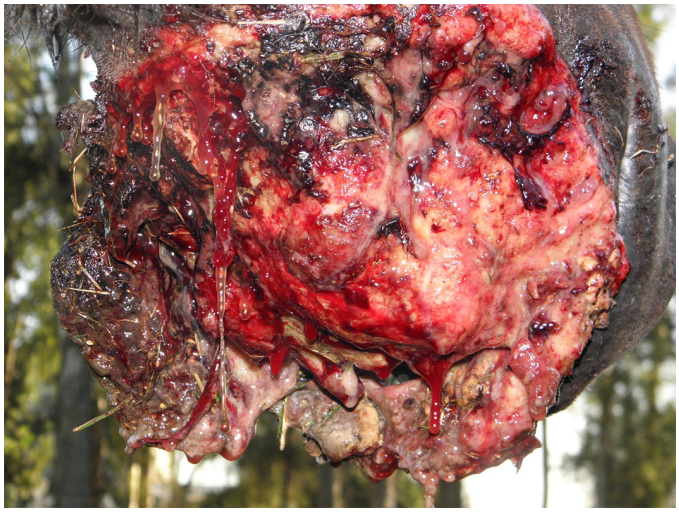


Fig.1. Case 1: Criollo mare. Ulcerated granulomatous growth in the lower lip and chin, with thick serosanguineous discharge.



Fig.2. Case 2: Arab mare. Extensive ulcerated tumor in the upper lip and nasal fossa, with numerous detached kunkers (arrow).

Table 1. Cases of equine pythiosis in the eastern region of Uruguay (2012–2017)

Case	Category	Breed	Age	Department	Place	Lesion location
1	Mare	Criollo	19 years	Treinta y Tres	Merin wetland	Lower lip, chin
2	Mare	Arab	5 years	Treinta y Tres	Merin wetland	Upper lip, nostrils
3	Stallion	Criollo	4 years	Treinta y Tres	Merin wetland	Left forelimb, fetlock
4	Stallion	Criollo	9 years	Rocha	Merin wetland	Right hindlimb, pastern
5	Mare	Criollo	12 years	Treinta y Tres	Merin wetland	Ventral abdomen
6	Filly	Criollo	7 months	Durazno	Negro River wetland	Right forelimb, cannon
7	Mare	Criollo	7 years	Treinta y Tres	Merin wetland	Right hindlimb, pastern and fetlock

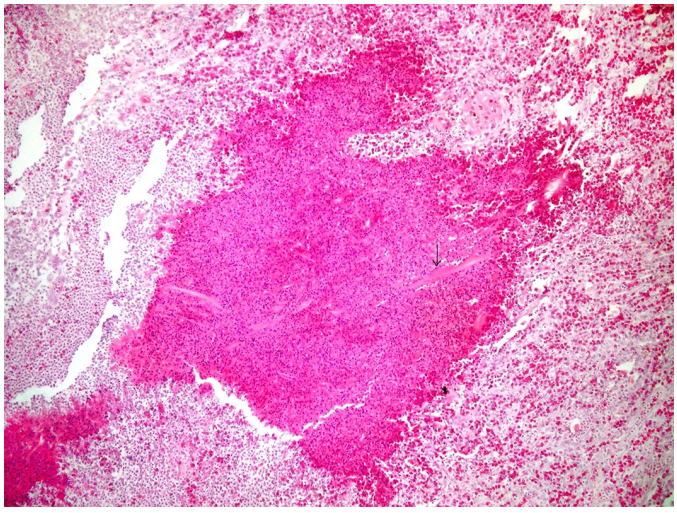


Fig.3. Case 3: Arab mare. Eosinophilic granuloma (kunker) with central amorphous necrosis and borders of macrophages and eosinophils in palisade. Degenerated collagen fibers are observed within the amorphous content (arrows). HE, obj.10x.

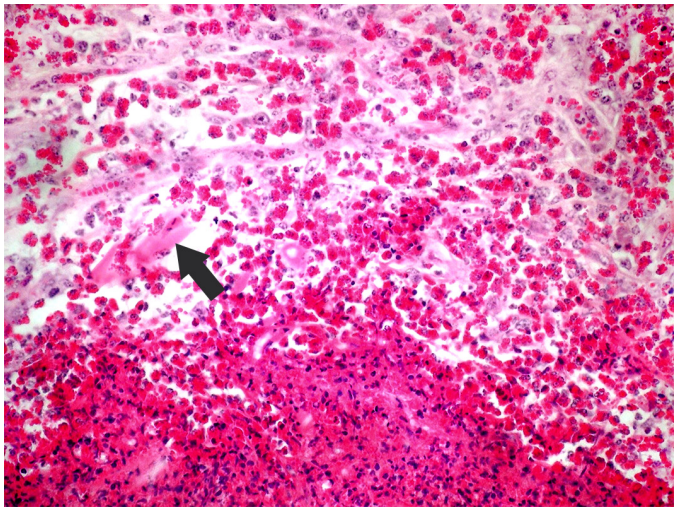


Fig.4. Case 3: Arab mare. Kunker edge showing a focus of amorphous necrosis (bottom-left), surrounded by numerous degranulated eosinophils around degenerated collagen fibers (top-right). HE, obj.40x.

the margins of the wound showed a rapid re-epithelization (Fig.6B,C). After 1 year, the wound was almost completely healed by secondary intention and the animal was discharged in very good body condition (Fig.6D).

Geographic pattern

The superposition of the topographic layer showed that six of the seven cases were located in the lower plains of the Merin lagoon basin in the departments of Cerro Largo, Treinta y Tres and Rocha, in eastern Uruguay, and the remaining case was in the Negro River fluvial plains, on the other side of the Cuchilla Grande hill range (200-500m altitude) that

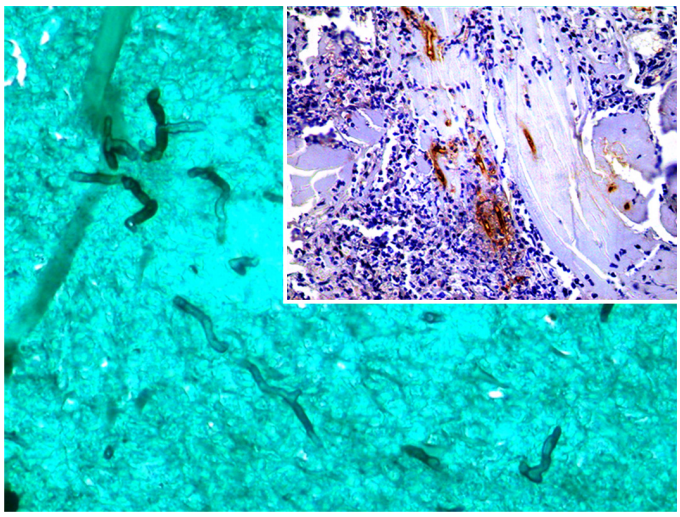


Fig.5. Case 7: Criollo mare. Non-septate intralesional hyphae, with discrete dilatations, and branching at 90º (GMS). Inset: Immunostaining for *Pythium insidiosum* hyphae by IHC, obj.100x.

defined the western limit of the basin (Fig.7A). The soils corresponded to the Vergara (1 case), Río Branco (2 cases), Rincón de Ramírez (2 cases), San Luis (1 case) units in the eastern plains, and the Río Tacuarembó unit (1 case) on the coasts of the Tacuarembó River. The drainage and inundation geographic layers (Fig.7B,C) showed that all these areas are characterized by poor or very poor natural drainage (Classes 6, 7 and 8) and are annually exposed to long or very long flooding, or remain flooded most of the year (Molfino & Califra 2004).

DISCUSSION

The epidemiology and the clinical, gross and histopathological findings in the seven studied horses were highly suggestive of *Pythium insidiosum* infection, which was confirmed by IHC in three animals. This technique is an effective and advantageous method when culture or isolation of the agent cannot be performed (Pedroso et al. 2009, Grooters 2013), but has the disadvantage that, because the hyphae are only found within the kunkers or into Splendore-Hoeppli foci (Mendoza et al. 1996), tissue sampling must be thorough. It had been observed that, in advanced stages of the disease, the exuberant formation of fibroplasia and granulation tissue made focal areas of necrosis distant from one another, making it difficult to obtain biopsies containing a sufficient quantity of kunkers for IHC diagnosis (Mendoza et al. 1996, Reis & Nogueira 2002). This is the reason for recommending the collection of several biopsies at different locations in the damaged tissue (Reis & Nogueira 2002). Thus, the absence of IHC-positive hyphae in some cases in the present study may be explained by the occurrence of an excessive proliferation of scar tissue in horses sampled in an advanced stage of the disease or, as samples came from routine material, biopsies did not include enough areas of necrosis where the hyphae are mostly visualized.



Fig.6. Case 7: Criollo mare treated with triamcinolone acetonide 50mg every 15 days. (**A**) Before starting treatment, (**B**) at 45 days, after 3 doses, (**C**) at 90 days, after 5 doses and (**D**) 1 year later.

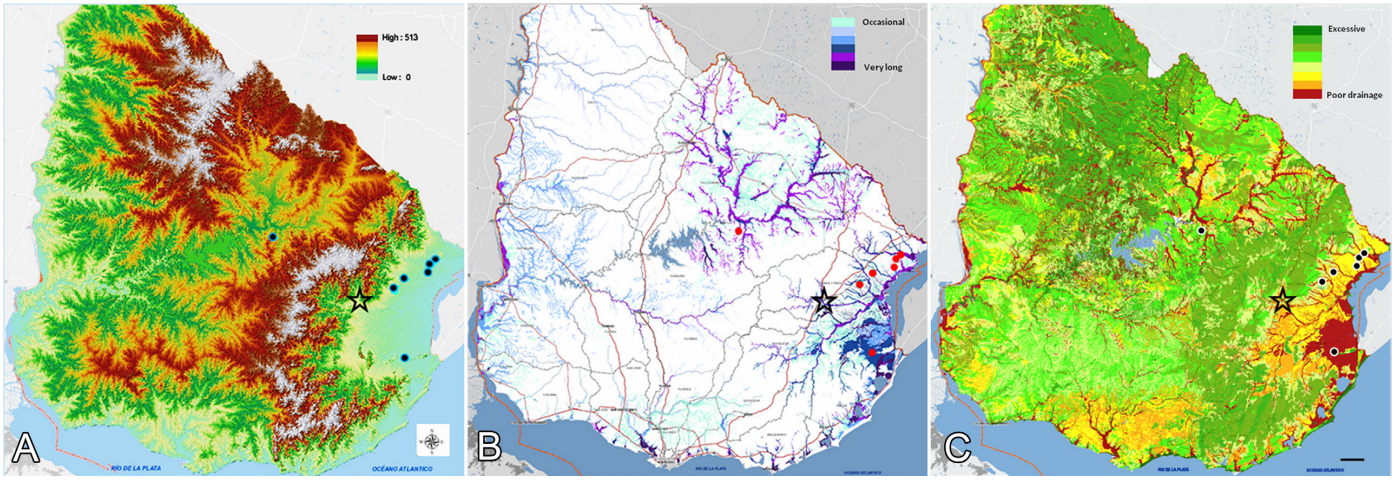


Fig.7. Geographic pattern of equine pythiosis in Uruguay. One-to-one point map with the following geographic layers superposed: (**A**) topographic layer (0 to 514 meters above sea level), (**B**) flooding layer (occasional to very long floodings), and (**C**) soil drainage layer (excessive to poor drainage). One point = one case. Maps obtained from <https://dgrn.mgap.gub.uy/js/visores/snia/#>

In the differential diagnosis, cutaneous zygomycosis such as basidiobolomycosis (*Basidiobolus ranarum*) and conidiobolomycosis (*Conidiobolus* spp.), which cause cutaneous granulomatous lesions similar to equine pythiosis, should be mainly considered (Grooters 2013, Mauldin & Peters-Kennedy 2015). Differentiation is important because the treatments and prognoses are different. In basidiobolomycosis, granulomatous lesions are most often found on the lateral aspects of the body due to the higher likelihood of these areas coming into contact with spore-containing soil or organic material when the animal lies on the ground, whereas conidiobolomycosis is mostly a nasopharyngeal infection, with or without dissemination to the external nares and face (Grooters 2013). In both diseases, kunkers are present, but are typically smaller (<2mm) and lack the discrete, branched corallike shape of pythiosis (Miller & Campbell 1984, Mauldin & Peters-Kennedy 2015). Microscopically, the diameter of the hyphae is significantly larger in *Basidiobolus* sp. and *Conidiobolus* sp. than in *P. insidiosus*, making them easily located on HE and PAS-stained histological sections (Miller & Campbell 1984). In addition, right-angled hyphae are considered typical of *P. insidiosum* (Mendoza et al. 1996). Thus, the pathological and histopathological findings of the present cases are all indicative of *Pythium insidiosum* infection.

The successful use of triamcinolone acetonide has previously been reported in the treatment of equine cutaneous pythiosis (Cuero-Castilla & Mendoza-Espinoza 1985 *apud* Mendoza et al. 1996, Cardona-Álvarez et al. 2016). Our experience with only one horse in the final phase of the disease confirmed these results. As surgical removal would be ineffective and pythiosis vaccines have not yet been approved in Uruguay, we decided to administer 50 mg IM triamcinolone acetonide biweekly,

as previously recommended (Cardona-Álvarez et al. 2016). The treatment began practically in the final phase of the disease when the animal was cachectic and very weak, and despite the large granulomatous ulceration around a distal leg, re-epithelization practically closed the wound after 1 year. The reasons for this potent curative effect of triamcinolone acetonide in the case of equine cutaneous pythiosis are uncertain. Unfortunately, we did not histologically follow the healing process. Triamcinolone acetonide inhibits eosinophil migration, reduces blood and tissue eosinophilia, and significantly enhances spontaneous eosinophil apoptosis (Zhang et al. 2000, Kankaanranta et al. 2005). In horses, triamcinolone is commonly used for the relief of itching and inflammation associated with a wide variety of eosinophilic skin conditions, such as eosinophilic granuloma, insect bite hypersensitivity, eosinophilic axillary nodular necrosis, habronemiasis, and even mastocytoma, among others (Scott & Miller Junior 2011). It has been proposed that exoantigens of *P. insidiosum* trigger a TH2 immune response as an evolutionary strategy for recruiting eosinophils and mast cells; these may worsen inflammation and secure the proliferation and survival of hyphae inside the eosinophilic necrosis foci (kunkers). On the other hand, cytoplasmic immunogens stimulate a protective TH1 response and the recruitment of macrophages and lymphocytes, which may result in death of the organisms and clinical remission (Mendoza & Newton 2005). Therefore, the high efficacy of triamcinolone for *P. insidiosum* infection in horses could be explained by its ability to modulate the immune response or inhibit the overwhelming number of degranulated eosinophils, limit the eosinophil-mediated tissue necrosis, decrease hyphae viability, and promote a rapid resolution of the eosinophilic inflammation. Other therapeutic studies are necessary, but our results suggest that an early histological diagnosis and treatment with triamcinolone acetonide would prevent the death of many horses in the endemic area.

In the present study, almost all cases of pythiosis occurred in the lower plains of the Merin lagoon basin, the largest wetland area in Uruguay (wetlands represent 4.5% of Uruguay´s area), which is transboundary with the counties of Rio Grande do Sul, Brazil, where equine pythiosis is clinically and serologically highly prevalent (Marcolongo-Pereira et al. 2012, Weiblen et al. 2016). Agroecologically, the eastern wetland is characterized by spring-summer growing grassland, rice-growing or rice-livestock farming systems, a high density of grazing horses (>3.08/sq km; OIE 2017), a humid subtropical climate and a dense network of rivers and small streams where soil humidity is permanent during a large part of the year and hydrophilic vegetation has adapted to recurrent or permanent flooding (Clara & Maneyro 1999); these environmental conditions are all ideal for the occurrence of equine pythiosis. *Pythium insidiosum* is a generalist pathogen, and ecological niche models predict its potential circulation through large areas in Rio Grande do Sul, Brazil, and Uruguay (Machado et al. 2018). In the present study, one case occurred in the fluvial wetlands of the Negro/Tacuarembó Rivers, but the disease might occur in other riverine wetlands of the country, such as the Carrasco/Santa Lucia plains (south of the country), lower reaches of the Uruguay River (Farrapos wetlands, southwest), or Queguay, Arapey and Dayman Rivers (northwest; Dr. Deborah César & Rodolfo Rivero, pers. comm. 2018), although horse density is much lower in these areas (<1.05/sq km; OIE 2017). Thus, the eastern wetlands appear to be the endemic ecological niche of equine pythiosis in Uruguay, although sporadic cases may occur in marshes and river plains in other parts of the country.

CONCLUSION

Pythiosis by *Pythium insidiosum* is an endemic disease of grazing horses in the wetland ecosystem of eastern Uruguay. Early treatment with triamcinolone acetonide is promising, but requires more research.

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Diagnosis of *Brachyspira pilosicoli, Brachyspira hyodysenteriae* and *Brachyspira intermedia* in hens and laying hens in the western region of Paraná through bacterial isolation and identification in qPCR[1]

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ABSTRACT.- Goulart T., Gruchouskei L., Gonçalves J., Cavasin J.P., Matos M.R., Faccin M & Viott A.M. 2019. **Diagnosis of *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* and *Brachyspira intermedia* in hens and laying hens in the western region of Paraná through bacterial isolation and identification in qPCR.** *Pesquisa Veterinária Brasileira 39(7):476-480.* Laboratório de Patologia Veterinária, Universidade Federal do Paraná, Setor Palotina, Av. Pioneiro 2153, Palotina, PR 85450-000, Brazil. E-mail: viott@ufpr.br

Bacteria of the genus *Brachyspira* can cause enteric diseases in poultry causing a decrease in productivity. The occurrence of this disease in chickens has already been verified in countries such as Australia, Italy, and the United States, but in Brazil, until now, epidemiological studies about *Brachyspira* sp. frequency were only carried out on pig farms. The objective of this study was to evaluate the presence of bacteria of the genus *Brachyspira* sp. through isolation and confirmation of the species *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* and *Brachyspira intermedia* using the qPCR technique. Samples from 110 hens aged from 35 to 82 weeks were collected, 40 were from commercial egg farms and 70 were from laying hens matrices. For the first evaluation, bacterial isolation was performed from the feces. Positive samples were submitted to qPCR to identify the three species proposed. Cecum fragments of the birds were collected and fixed in formaldehyde for histological evaluation and counting of goblet cells. Of the 110 samples, 48 characteristic isolates of *Brachyspira* (43.6%) were obtained and of these in qPCR 13 identified as *B. hyodysenteriae* (11.8%) and 5 all from the same farm as *Brachyspira intermedia* (4.5%), 2 samples were positive for both agents (1.8%) and 28 were not characterized by qPCR (25.5%). None histopathological lesions were observed in the chicken cecum and no significant statistical difference was noticed in the count of goblet cells of the positive hens. It can be evidenced by the occurrence of *Brachyspira s*p. in laying farms and hens in Brazil, with special relevance to *Brachyspira intermedia* that can be potentially pathogenic for these animals.

INDEX TERMS: *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae*, *Brachyspira intermedia*, hens, laying hens, Paraná, bacterial isolation, qPCR, commercial birds, frequency, enteropathogens, spirochetosis, bacterioses.

RESUMO.- [**Diagnóstico de *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae* e *Brachyspira intermedia* em aves de postura e matrizes de corte na região oeste do Paraná através do isolamento bacteriano e identificação na qPCR.**] Bactérias do gênero *Brachyspira* podem ocasionar enfermidades entéricas em aves acarretando a queda de produtividade. A ocorrência desta enfermidade em galinhas já foi verificada em países como a Austrália, Itália e Estados Unidos, porém no Brasil, até o momento, trabalhos epidemiológicos sobre a frequencia de *Brachyspira* sp. só foram realizados em granjas de suínos. O objetivo deste estudo foi avaliar a presença de bactérias do gênero *Brachyspira* sp. através do isolamento e confirmação das espécies *Brachyspira pilosicoli, Brachyspira hyodysenteriae* e *Brachyspira intermedia* utilizando a técnica de qPCR. Foram coletadas amostras de 110 aves com idade

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entre 35 e 82 semanas, sendo 40 de granjas de postura comercial e 70 de granjas de matrizes de corte. Para avaliação primeiramente procedeu-se o isolamento bacteriano a partir das fezes. As amostras positivas foram submetidas a qPCR para identificação das três espécies propostas. Fragmentos de ceco das aves foram coletados e fixados em formol para avaliação histológica e contagem de células caliciformes. Das 110 amostras foram obtidos 48 isolamentos característicos de *Brachyspira* (43,6%) e destes na qPCR 13 identificadas como *B. hyodysenteriae* (11,8%) e 5 sendo todas da mesma granja (4,5%) como *B. intermedia*, 2 amostras foram positivas para ambos os agentes (1,8%) e 28 não foram caracterizadas através da qPCR (25,5%). Não foram observadas alterações histopatológicas no ceco e diferença estatística significativa na contagem de células caliciformes das aves positivas. Conclui-se que a *Brachyspira* sp. é frequente em granjas de poedeiras e matrizes de corte no Brasil, com especial relevância para a *B. intermedia* que possui potência patogênico para estas aves.

TERMOS DE INDEXAÇÂO: *Brachyspira pilosicoli*, *Brachyspira hyodysenteriae*, *Brachyspira intermedia*, aves de postura, matrizes de corte, Paraná, isolamento bacteriano, qPCR, aves comerciais, frequência, enteropatogenos, espiroquetose, bacterioses.

INTRODUCTION

Bacteria of the *Brachyspira* species can cause enteric diseases, especially in birds and swine, besides that, they have zoonotic feature. Four species of *Brachyspira* are considered pathogenic to birds, being *Brachyspira intermedia, Brachyspira pilosicoli*, *Brachyspira alvinipulli* and *Brachyspira hyodysenteriae,* and one or more species can be concomitantly involved in the development of diseases (Song & Hampson 2009, Mappley et al. 2014).

On hens, the *Brachyspira* sp. is associated with a clinical condition known as avian intestinal spirochetosis (AIS) commonly observed in older birds (Phillips et al. 2005, Medhanie et al. 2013, Mappley et al. 2014). *B. intermedia* and *B. pilosicoli* are the main causers (Stephens & Hampson 2002, Bano et al. 2008), and *B. alvinipulli* appears in lower frequency (Phillips et al. 2006, Feberwee et al. 2008). The clinical signs include chronic diarrhea, weight loss, low egg productivity, and eggs dirty with feces (Medhanie et al. 2013). Burch et al. (2006) compared the performance of lots with AIS and the standard for the laying lineage and found a 6% decrease in egg production and an 8.84% increase in the mortality rate in an untreated lot.

B. hyodysenteriae is associated with the occurrence of severe typhlitis in rheas (*Rhea americana*) naturally infected, being the same spirochete that causes swine dysentery (Jensen et al. 1996), disease that has been causing major losses in the Brazilian swine culture (Garcia 2015). Hens are natural carriers of this species, but in these birds *B. hyodysenteriae* is apathogenic (Feberwee et al. 2008). Nevertheless, this *Brachyspira* species has importance in poultry and swine producing regions, as observed by Backhans et al. (2011) *Brachyspira* sp. can transit between birds, pigs and rodents. All these animals can be colonized from a common environmental source, so rats and mice can act on the transmission of *B. hyodysenteriae* and *B. pilosicoli* among lots or even granges of different species.

Occurrence of *Brachyspira* sp. has already been found in poultry-producing countries such as Italy where *Brachyspira* was 72.4% in laying granges and 31% of these samples were pathogenic (Bano et al. 2008). In Australia the prevalence found was 42% in broilers and 68% in laying hens (Stephens & Hampson 2002); and recently in Argentina, 44% positivity was found in the evaluated laying hens (Illanes et al. 2016).

Due to the lack of data on the occurrence of AIS in Brazil, this study evaluated for the first time the occurrence of *Brachyspira* sp. in commercial birds and broiler breeders in western Paraná State, through selective anaerobic culture, and isolates were identified using the qPCR technique for *B. pilosicoli*, *B. intermedia* and *B. hyodysenteriae* species.

MATERIALS AND METHODS

Samples. This study was approved by the Animal Use Ethics Committee of the Pallottine Sector of the "Universidade Federal do Paraná" (UFPR) under the protocol CEUA-Palotina 06/2017. One hundred and ten samples were collected, all in farms located in western Paraná. Forty samples of hens kept in cage were collected from two commercial poultry farms located in the district of Céu Azul, of the Californian style. One of the properties had four floors of cages and the birds were 82 weeks old, the other farm had no fences, only one floor of cages and birds were aged 35 and 40 weeks. This last property had also a pig grange. The other 70 samples were from broiler breeders coming from two breeders that had a rigorous biosafety scheme with separate nuclei and climate-controlled houses with a density of seven birds per m^2. Of these, 20 samples were from the Assis Chateaubriand city broiler, divided between two nuclei aged 47 and 59 weeks. The remaining 50 samples were from a Palotina city breeder, divided into five nuclei aged 46, 60, 65 and 67 weeks; one of the nuclei aged 60 weeks had a history of diarrhea at week 46. The birds were randomly selected throughout the house and then necropsied. Cecum segments were collected and immediately packed in an ice-cold isotherm box and sent to the laboratory for bacterial isolation. Cecum fragments were collected and fixed in 10% buffered formalin and further processed by the routine paraffin embedding technique recommended by Tolosa et al. (2003) for periodic acid-Schiff (PAS) and hematoxylin-eosin (HE) staining.

Bacterial isolation. The protocol for bacterial isolation followed the recommendations by Neves (2012). Feces samples were seeded by plating with *Brachyspira* sp. (anaerobiosis agar (Neogen Co, MI, USA), 5% sheep blood, 6.25mg/µl rifampicin (Sigma-Aldrich Co, MO, USA), 800mg/µl of spectinomycin (Sigma-Aldrich Co, MO, USA), 25mg/µl of vancomycin (Sigma-Aldrich Co, MO, USA), 25mg/µl of colistin (Sigma-Aldrich Co, MO, USA). They were incubated in anaerobiosis jar with an anaerobic atmosphere generated with anaerobiosis media (Anaerobac®, Probac of Brazil, São Paulo, Brazil) at 42°C for three days or until evidence of hemolysis. The anaerobic environment was confirmed by an anaerobiosis indicator strip (Oxoid Anaerobic Indicator®, Thermo Fisher, MA, USA). Growth was considered positive when areas of strong and weak plaque hemolysis were evidenced; it was sometimes accompanied by white millimeter colonies, suggesting *Brachyspira* sp. These areas and/or colonies were carefully picked using a calibrated loop on anaerobic isolation agar plates (Neogen Co, MI, USA) containing 5% sheep blood and incubated anaerobically for three days at 37°C. W. After obtaining *Brachyspira* sp. these were collected with a calibrated loop and resuspended in 1.5mL of fetal bovine serum. This suspension was frozen at -20°C until qPCR was performed.

qPCR (real time polymerase chain reaction). DNA was extracted from all samples that showed growth for *Brachyspira* sp.

using QIAamp cador Pathogen Mini Kit® (Qiagen, New York, USA) according to manufacturer's instructions.

Samples were analyzed in a private laboratory using primers described by La et al. (2003) for *B. hyodysenteriae* and *B. pilosicoli*, and for *B. intermedia* the sequence described by Phillips et al. (2005). The protocol followed the recommendations of Song & Hampson (2009).

Histopathology. HE-stained cecum sections were evaluated for possible histological lesions compatible with *Brachyspira* infection such as inflammatory changes, epithelial erosion, intralesional bacteria, "false brush border" formation, crypt hyperplasia and lymphoid follicle hyperplasia.

PAS and goblet cell count. 5µm thick sections of cecum fragments were subjected to PAS staining to evidence the goblet cells.

For the goblet cell count, the cecum sections were analyzed under the microscope and the number of cells from ten random fields were counted, the marked goblet cells were accounted and from these values an average was obtained according to Gottardo et al. (2016).

Statistical analysis. The averages of bird goblet cells in isolation positive, in isolation positive with confirmation of species by *B. hyodysenteriae* qPCR and in isolation positive with confirmation of species by qPCR for *B. intermedia* were each compared separately with the group negative in bacterial isolation. The chi-square test was performed, considering $p<0.05$ statistically significant, using SAS 9.0 software.

RESULTS AND DISCUSSION

One hundred and ten samples were analyzed, 40 from commercial layers and 70 from matrices. Forty-eight samples were positive for *Brachyspira* sp. in isolation, being positive 47.5% of the commercial laying hens and 41% for the matrices, totalizing 43.6% of the samples. This result was very close to that found by Illanes et al. (2016) in Argentina in laying hens; however, lower than the 68% found in Australia by Stephens & Hampson (2002) or the 72.4% in Italy by Bano et al. (2008).

Between the two pathogenic species that were identified by the qPCR technique, no sample was characterized as *Brachyspira pilosicoli*, a species of special importance for being zoonotic and already detected in birds in Argentina (Illanes et al. 2016). The birds analyzed were not on the effect of antibiotics such as tiamulin and lincomycin, pointed out by Stephens & Hampson (2002) as effective against *B. intermedia* and *B. pilosicoli*, which could have influenced bacterial isolation. However, the fact that the medium used contains vancomycin could have influenced the non-isolation of *B. pilosicoli*, as Calderaro et al. (2005) have already pointed it out as a possible growth inhibitor of *B. pilosicoli* in swine. Five samples were identified as *B. intermedia*, all from the same commercial laying hen grange, which corresponded to 10.5% of *Brachyspira* isolates, a percentage also lower than that found in Italy by Bano et al. (2008), showing a lower incidence of AIS agents in Brazil. These results are summarized in Table 1.

Among the farms analyzed, only one of commercial posture showed no positive sample of *Brachyspira* sp. through culture, the birds of this farm were 82 weeks old. Colonization by *Brachyspira* sp. is significantly larger in older birds, suggesting that after initial contact the bacterial population increases over time (Phillips et al. 2005, Medhanie et al. 2013). Even though the free grange is the one with the older birds analyzed, this may be associated with the good sanitary control measures adopted in the farm, which prevented the agent from entering and spreading.

However, in another commercial broiler farm with 35 and 40-week-old birds, only one in 20 birds was not positive at the age of 35 weeks. In the study by Medhanie et al. (2013), who assessed risk factors for colonization of *Brachyspira* sp. in hens, the presence of birds of different ages in the same farm indicates a higher probability of finding positive birds for *Brachyspira* sp., which could be observed in this farm.

Twenty-eight samples (58.4%) positive in isolation were not characterized among the three species surveyed; a possible cause is due to the large number of apathogenic *Brachyspira* species already identified in the bird intestine, including *B. innocens*, *B. murdochii* and *B. pulli* (Feberwee et al. 2008). Among the pathogenic species described in birds, *B. alvinipulli* is reported in low field frequency, with few isolations and outbreaks reported in the literature (Phillips et al. 2005, Feberwee et al. 2008), not being the target of this work.

In this study, as found by Phillips et al. (2005) and Illanes et al. (2016), there were positive birds raised in the cage system. Since the transmission route of *Brachyspira* sp. is due to fecal-oral contact, in this type of system birds have less exposure to excreta, which could reduce contamination, but birds can also be exposed to feces through flies and rodents (Medhanie et al. 2013). Among the 48 samples submitted to qPCR, two (4.1%), both from a commercial laying farm, presented positive samples for both *B. hyodesenteriae* and *B. intermedia.* On this property, the California-type aviary had no barriers to the entry of wild and domestic animals, and the place had a pig grange.

Colonization by *B. hyodysenteriae* was found in 15.7% of the positive laying hen samples and in 35% of the hens. In hens, the natural colonization by this species had already been verified by Feberwee et al. (2008). Although this species is not associated with the occurrence of AIS, intestinal infection by *B. hyodysenteriae* in swine can cause severe high-impact mucus-hemorrhagic diarrhea called swine

Table 1. Number of positive culture samples for *Brachyspira* sp. of poultry and commercial breeders and identification of *Brachyspira hyodysenteriae, B. pilosicoli* and *B. intermedia* species by qPCR techniques

	Positive by isolation	qPCR* *B. hyodysenteriae*	qPCR* *B. pilosicoli*	qPCR* *B. intermedia*	qPCR* *B. intermedia*+ *B.hyodysenteriae*	Not characterized by qPCR*
Commercial layers	19/40 (47.5%)	3/19 (15.7%)	0/19 (0%)	5/19 (26.3%)	2/19 (10.5%)	9/19 (47.5%)
Matrix	29/70 (41%)	10/29 (35%)	0/29 (0%)	0/29 (0%)	0/29 (0%)	19/29 (65%)
Commercial layers+Matrix		13/48 (27%)	0/48 (0%)	5/48 (10.5%)	2/48 (4.1%)	28/48 (58.4%)
Total	48/110 43.6%	13/110 (11.8%)	0/110 (0%)	5/110 (4.5%)	2/110 (1.8%)	28/110 (25.5%)

* qPCR real time polymerase chain reaction.

dysentery (Boye et al., 1998), a disease already evident in the region of this study (Garcia 2015). Thus, the positivity of samples for *B. hyodysenteriae*, 13% of all birds analyzed, should serve as a warning due to the proximity of swine and poultry granges in regions where agroindustry is prevalent. As verified by Backhans et al. (2011), swine, poultry and rodents may carry the same *Brachyspira* species and serve as a source of dissemination.

The hemolysis evidenced in the cultures was of strong hemolysis for the 15 samples identified in the qPCR as *B. hyodysenteriae*, a striking feature of the species in the isolation (Mappley et al. 2014). Strong hemolysis was also observed in five samples identified as *B. intermedia*, which are routinely classified as having poor hemolysis similar to that previously reported by McLaren et al. (1997) in commercial layers such as this study. Among the 28 samples not identified by qPCR, 22 presented poor hemolysis, but six were strongly hemolytic: five from the same matrix nucleus and a commercial laying sample. Strong hemolysis, similar to the decay for *B. hyodysenteriae*, has already been observed in two new species, *B. suanatina* and *B. hampsonii*, which have also been identified in swine and wild birds (Råsbäck et al. 2007, Chander et al. 2012).

No histological changes compatible with *Brachyspira* sp. as verified by Stephens & Hampson (2002), who evaluated experimentally infected hens were verified. In this study, the lack of lesions was associated with the fact that the analysis was performed several weeks after inoculation and there was time for resolution of possible lesions. Even after recovery and reestablishment of lesions, birds can eliminate *Brachyspira* for long periods; *B. intermedia*, a pathogenic species isolated in the present study, was already detected in hens' excreta nine months after the challenge (Dwars et al. 1990). In the study by Feberwee et al. (2008), evaluating naturally infected animals, found mild inflammatory alterations, but the animals analyzed by them came from farms that already presented signs compatible with AIS. In addition, the evaluated animals had pre-existing enteritis by other agents, which may be a predisposing factor for the pathogenicity of *Brachyspira* sp. These conditions were not evidenced in the birds of this work, which may have motivated the lack of histological lesions.

The increase in the number of goblet cells is a change commonly observed in animals infected with *Brachyspira* sp. (Jensen et al. 1996, Shivaprasad & Duhamel 2005, Feberwee et al. 2008), but this change was not observed. Isolation negative birds were compared with three groups: isolation positive birds, positive isolated with species confirmation by *B. hyodysenteriae* and *B. intermedia* qPCR, in all comparisons goblet cell numbers showed no statistically significant changes. This fact can be explained by the lack of histological lesion in the intestine.

CONCLUSION

Bacteria of the genus *Brachyspira* are present in broiler farms and laying hens in Western Paraná, Brazil. Pathogenic strains for poultry such as *B. intermedia* and swine as *B. hyodysenteriae* can be isolated from healthy poultry excreta, serving as a source of contamination and dissemination of these agents.

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

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Carrier frequency of autosomal recessive disorders (BC, BLAD, FXID and CVM) in Holstein cows in Jalisco, Mexico[1]

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ABSTRACT.- Virgen-Méndez A., Ayala-Valdovinos M.A., Galindo-García J., Sánchez-Chiprés D.R., Lemus-Flores C. & Duifhuis-Rivera T. 2019. **Carrier frequency of autosomal recessive disorders (BC, BLAD, FXID and CVM) in Holstein cows in Jalisco, Mexico**. *Pesquisa Veterinária Brasileira 39(7):481-484.* Departamento de Producción Animal, Centro Universitario de Ciencias Biológicas y Agropecuarias, División de Ciencias Veterinarias, Universidad de Guadalajara, Camino Ramón Padilla Sánchez 2100, Nextipac, Zapopan, Jal. C.P. 44600, Mexico. Tel: 33 36821454; E-mail: manayala@cucba.udg.mx

The hereditary autosomal recessive disorders bovine citrullinemia (BC), bovine leukocyte adhesion deficiency (BLAD), factor XI deficiency (FXID), and complex vertebral malformation (CVM) have affected dairy cattle breeding significantly around the world. This study examined the carrier frequency of BC, BLAD, FXID, and CVM autosomal recessive disorders in *Bos taurus* Holstein cows bred in the Altos Norte region of the state of Jalisco, Mexico. We extracted DNA from 408 random samples of peripheral blood, and then used polymerase chain reaction (PCR) to identify insertion mutations for FXID, and PCR with restriction fragment length polymorphism (PCR-RFLP) for CVM, BC and BLAD. We visualized the PCR products using agarose gel electrophoresis stained with GelRed®. We found that 100% of wild-type (N/N) allele homozygous animals for genes *CD18*, *ASS*, and *FXI* were free of the mutations for BLAD, BC and FXID respectively. For gene *SLC35A3* we estimated total carrier frequency of 10.3% and allele frequency of 5%.

INDEX TERMS: Holstein cows, genetic disease, vertebral malformation complex (CVM), factor XI deficiency (FXID), bovine citrullinemia (BC), bovine leukocyte adhesion deficiency (BLAD), Mexico, bovine.

INTRODUCTION

Overbreeding of dairy cattle from a narrow genetic base has led to undesirable effects and genetic anomalies. Animals are chosen for breeding based on potential yield without taking into account reproductive and health criteria (Cole et al. 2016). Among the most well-known recessive genetic defects in Holstein cattle are complex vertebral malformation (CVM), bovine leukocyte adhesion deficiency (BLAD), factor XI deficiency (FXID), and bovine citrullinemia (BC). These conditions have led to significant drops in production and economic losses (Gholap et al. 2014, Cole et al. 2016).

CVM, which is caused by a missense mutation, a single-base transversion of guanine to thymine at position 559 in the gene *SLC35A3* on bovine chromosome 3, is characterized by delayed growth, vertebral malformation, and bilateral arthrogryposis leading to premature death in calves (Agerholm et al. 2001, 2004). BLAD is characterized by impaired migration of leukocytes to infection sites and tissue lesions during immune responses (Nasreen et al. 2009). The molecular basis of BLAD is a missense mutation (A to G) at position 383 of the *CD18* gene located bovine chromosome 1 (Nagahata 2004). FXID, which is caused by a mutation of *FXI* gene on bovine chromosome 27, is an insertion of a 76-bp adenine-rich fragment in exon 12. This insertion, composed of an imperfect poly-adenine tract followed by a duplicated region of the normal coding sequence, introduces a premature stop codon, which impairs the synthesis of functional protein (Marron et al. 2004), is associated with excessive bleeding and reproductive problems in Holstein

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cattle (Korkmaz Ağaoğlu et al. 2015). BC, which is caused by a single-base substitution (C to T) at codon 86, converting it to a nonsense codon of the gene coding of argininosuccinate synthetase *ASS* gene, on bovine chromosome 11 (Dennis et al. 1989), is characterized by hyperammonemia due to urea cycle interruption, which leads to neurological problems resulting ultimately in coma and death in the first week of life (Harper et al. 1986, Dennis et al. 1989).

In Mexico there are no studies on the prevalence of carriers for BC, FXID and CVM, and there is only one study on BLAD carrier frequency in the northeast region of the country (Riojas Valdés et al. 2009). Given the scarcity of information on frequency of these mutations in Holstein dairy herds in Mexico, the objective of our study was to determine the carrier frequency of genes associated with these recessive hereditary disorders in the Altos Norte region of the state of Jalisco, Mexico.

MATERIALS AND METHODS

Ethics statement. Laboratory analysis was done at the Institute of Animal Biotechnology of the University of Guadalajara, Mexico. The study was approved by Internal Bioethics Regulations of the University Center for Biological and Agricultural Sciences, University of Guadalajara, Mexico (Approval No. CC/NN11-12/00/2012).

Sample collection and DNA extraction. To calculate the size of the necessary sample, the methodology described by Fosgate (2009) was used, a total of 408 random samples were obtained from adult Holstein cows, aged between 3 and 6 years, with different physical condition and physiological state, in dairy herds of the Altos Norte region of the state of Jalisco. From each animal we collected 3ml of peripheral blood by caudal vein puncture into a Vacutainer® tube with EDTA. We extracted DNA from each blood sample using a Quick-DNA™ Universal Kit (Zymo Research, Orange/CA, USA).

Amplification. For PCR, we used Thermo Scientific™ PCR kits (Takara Bio Inc., Kusatsu/Shiga, Japan) with a 20µl reaction mix containing ~100ng blood lysate, 2µl PCR 1× buffer with 20mM $MgCl_2$, 1µl mix of 10mM dNTP, 0.5µl DreamTaq® DNA Polymerase, 5pmol of both primers, and the remaining volume double-distilled water (ddH_2O). We carried out PCRs on a Techne® TC-5000 Thermal Cycler (Techne Inc., Burlington/NJ, USA) using the primers and conditioners described above and shown in Table 1, to amplify the regions of interest in *SLC35A3*, *CD18*, *FXI* and *ASS*. For the verification of the PCR-RFLP analysis, we used as a control samples, commercial semen of straws from bulls that were genotyped for these genetic diseases. We analyzed the amplification products by electrophoresis in 4% agarose gel stained with GelRed™ (Biotium, Hayward/CA, USA) and visualized the results under ultraviolet light.

Estimation of genotype and allele frequencies for each gene. To estimate genotype and allele frequencies, we applied the direct counting method described by Nei (1987).

RESULTS

In the case of CVM, we identified 42 (10.3%) carrier cows (N/CVM) and 366 (89.7%) cows free of the mutation (N/N). The homozygous mutant (CVM/CVM) was absent due to the lethal nature of the disorder and the sample type (only adult cows). Allele frequencies for CVM were 5% with 95% wild-type allele. We found no carrier cows for BLAD, FXID, or BC; that is, 100% of the 408 animals were homozygous for the wild-type alleles of genes related to these disorders.

DISCUSSION

Since early in the 21st century, high frequencies of CVM carrier animals have been reported in countries such as Japan, at 32.5% (Nagahata et al. 2002), Sweden, at 23% (Berglund et al. 2004), and Poland, at 24.8% (Ruść & Kamiński 2007), leading several countries to develop programs aimed at reducing CVM carrier prevalence (Ghanem et al. 2008, Ruść et al. 2013). The prevalence of carrier animals in this study was estimated at 10%, was similar to that reported for Japan, at 13% (Ghanem et al. 2008), lower than that reported for Poland, at 16.6% (Ruść et al. 2013), and higher than in Brazil's Girolando cattle, at 1.5% (Paiva et al. 2013).

In the 1990s, different carrier frequencies for the BLAD-associated mutation were reported in a number of countries, including USA at 14.1% (Shuster et al. 1992), Denmark at 21.5% (Jørgensen et al. 1993), and Japan at 13.4% (Nagahata et al. 1997). Since 2000, there has been a marked decrease in the carrier rates, such as in Iran with 3.33% (Norouzy et al. 2005), China with 1.36% (Schifferli et al. 2000), and Turkey with 2% (Korkmaz Ağaoğlu et al. 2015). The non-incidence of BLAD carriers in our study is in agreement with reports from the Czech Republic (Citek et al. 2006) and Turkey (Oner et al. 2010).

FXID carrier frequency data have been reported in various parts of the world. In the USA, the reported frequency was 1.19% (Marron et al. 2004), in Japan 2.5% (Ghanem et al. 2005), in the Czech Republic 0.36% (Citek et al. 2008). In Turkey, FXID carrier frequency studies have been conducted in diverse regions. In the Bursa region (Oner et al. 2010), carrier

Table 1. Primers used for genotyping *SLC35A3, CD18, FXI* and *ASS* mutations

Gene	Genetic disorder	Primers (5'-3')	Tm (°C)	PCR product (bp)	GenBank access No.	RE	Reference
SLC35A3	CVM	F: 5'-CACAATTTGTAGGTCTCATGGCA-3' R: 5'-CGATGAAAAAGGAACCAAAAGGG-3'	54°C	233bp	AY160683	Pst I	Kanae et al. (2005)
CD18	BLAD	F: 5'-AGGCAGTTGCGTTCAACGTGA-3' R: 5'-CCGACTCGGTGATGCCATTGA-3'	53°C	159bp	M81233	Taq I	Schifferli et al. (2000)
FXI	FXID	F: 5'-CCCACTGGCTAGGAATCGTT-3' R: 5'-CAAGGCAATGTCATATCCAC-3'	54°C	244bp	AH013749	N/A	Marron et al. (2004)
ASS	BC	F: 5'-GGCCAGGGACCGTGTTCATTGAGGACATC-3' R: 5'-TTCCTGGGACCCCGTGAGACACATACTTG-3'	51°C	185bp	AC000168	Ava II	Dennis et al. (1989)

CVM = Complex vertebral malformation, BLAD = bovine leukocyte adhesion deficiency, FXID = factor XI deficiency, BC = bovine citrullinemia, F = forward, R = reverse, Tm = melting temperature, PCR = polymerase chain restriction, bp = base pairs, RE = restriction enzyme, N/A = not applicable.

frequency of 1.17% was found, in the Burdur region it was 1.8% (Korkmaz Ağaoğlu et al. 2015), and in the Thrace region no FXID carriers were found (Avanus & Ahmet 2016), as in the present study. However, for FXID, besides the number of animals sampled, the choice of random or deliberate selection criteria may also be related to the variability in FXID prevalence rates between different countries. None of these previous studies considered repeat breeding syndrome in cows as important to the selection of animals for genotyping. Thus, as suggested by Avanus & Ahmet (2016), if future studies sample animals with this syndrome preferentially, the FXID allele frequency might increase.

The BC condition was reported for the first time by Healy et al. (1990) after importing semen from the US progenitor Linmack Kriss King for the Holstein population in Australia. There were subsequent reports of BC carrier prevalence in various countries. Whereas the reported carrier frequency in the USA was 0.3% (Robinson et al. 1993), it was 13% in Australia, the highest reported prevalence (Healy 1996). Generally, carrier frequencies are very low, as in the case of China with 0.16% (Li et al. 2011), or null as we found here, which was also reported in Germany (Grupe et al. 1996), Czech Republic (Citek et al. 2006), India (Patel et al. 2006), and Turkey (Korkmaz Ağaoğlu et al. 2015). While the FXID, BC, BLAD, and CVM related alleles have been reported in various countries, in Mexico there are no previous studies of these mutations, except a single study for BLAD (Riojas Valdés et al. 2009). In our study of 408 Holstein cows in the Altos Norte region of Jalisco for all these recessive disorders (BC, BLAD, FXID and CVM), we confirmed only the presence of CVM carriers (10.3%). A considerable prevalence of a recessive lethal defect such as CVM could be caused by a high level of inbreeding in livestock populations, by the lack of knowledge of the possible existence of CVM by the livestock producers in cases of neonatal mortality (Ghanem & Nishibori 2008), and by the absence of marker-assisted programs that could substantially reduce the frequency of a mutation within a little period. The different selection strategies against the lethal recessive allele in CVM are reflected in the different reduction rates of the specific allele frequencies (Schütz, et al. 2008). The absence of the mutant alleles for the genetic disorders BLAD, FXID, and BC may be due to the fact that most Holstein cattle germoplasm in Mexico is imported from the USA and Canada, where companies in charge of bovine genetics have in place selection programs to combat these hereditary diseases (Nagahata 2004) with positive effects for the cattle industry in Mexico. Despite the efforts of other countries to counteract the impact of these harmful mutations, the presence of the CVM-causing allele in our study points to insufficient planning and genetic selection of reproductive animals in Mexico.

CONCLUSIONS

In this study, we found no evidence of mutant alleles for the recessive hereditary disorders BC, BLAD, and FXID in Holstein cows in the Altos Norte region of the state of Jalisco.

For the *SLC35A3* mutation associated with the hereditary CVM disorder, we calculated a mutant allele frequency at 5% and a carrier frequency at 10%.

This study seems to be the first to report the allele frequency of FXID, CVM, BLAD and BC in Holstein cattle located in Jalisco, Mexico.

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

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Clinical and blood gas analysis of calves conceived by artificial insemination, *in vitro* fertilization and animal cloning[1]

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ABSTRACT.- Dantas G.N., Santarosa B.P., Benesi F.J., Santos V.H. & Gonçalves R.C. 2019. **Clinical and blood gas analysis of calves conceived by artificial insemination, *in vitro* fertilization and animal cloning.** *Pesquisa Veterinária Brasileira 39(7):485-491.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Distrito de Rubião Júnior s/n, Botucatu, SP 18618-970, Brazil. E-mail: gabrielan.dantas@gmail.com

In order for successful extra-uterine adaptation to occur, it is necessary for the neonate to be able to establish its respiratory functions effectively, guaranteeing efficient oxygenation and good vitality. Respiratory disorders are the major cause of death during the neonatal period in cattle, and this mortality is even more significant when it comes to calves originated by *in vitro* fertilization (FIV) or animal cloning (CA). Blood gas analysis assesses acid-base balance changes effectively, and when associated with the neonate's clinical examination, provides subsidies for accurate diagnosis and early treatment of neonatal maladaptation. The objective of this study was to study neonates born from artificial insemination (IA) and to compare them to calves conceived by FIV and CA, regarding blood gas and clinical examination. For that, 20 AI calves, 15 FIV calves, and 15 cloned calves were evaluated immediately after calving and at 6, 12, 24 and 48 hours of life. At all experimental times, venous blood samples were collected for blood gas and clinical examination was performed. In the postpartum evaluation, Apgar score and column length and respiratory amplitude measurements were used. IVF animals showed no alterations, resembling Group IA calves. The calves from CA showed more pronounced acidosis postpartum than expected physiological acidosis mixed for neonates, with decreasing values of bicarbonate (HCO_3^-), and base excess (BE) and the increase in carbon dioxide pressure (PCO_2) when compared to the other groups. This disorder may have reflected lower mean values of Apgar scores and increased heart and respiratory rates. Intensive follow-up of these neonates is suggested, with monitoring by clinical and hemogasometric examination for early diagnosis of this condition and treatment based on oxygen therapy and bicarbonate replacement.

INDEX THERMS: Clinics, blood gas, calves, analysis, artificial insemination, *vitro* fertilization, animal cloning, apgar score, cattle, neonatology, respiratory acidosis, respiratory amplitude.

RESUMO.- [**Análise clínica e hemogasométrica de bezerros concebidos por inseminação artificial, fertilização *in vitro* e clonagem animal.**] Para que ocorra adaptação extra-uterina bem sucedida é necessário que o neonato consiga estabelecer suas funções respiratórias de maneira eficaz, garantindo oxigenação eficiente e boa vitalidade. Distúrbios respiratórios são os maiores causadores de óbito durante o período neonatal em bovinos, e essa mortalidade é ainda mais expressiva quando se trata de bezerros originados por fertilização *in vitro* (FIV) ou clonagem animal (CA). A hemogasometria avalia alterações do equilíbrio ácido-básico de forma eficaz,

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e quando associada ao exame clínico do neonato, fornece subsídios para diagnóstico acurado e tratamento precoce da má adaptação neonatal. O objetivo deste trabalho foi estudar recém-nascidos bovinos originados por inseminação artificial (IA) e compará-los a bezerros concebidos por FIV e CA, no que se refere a hemogasometria e exame clínico. Para isso, foram utilizados 20 bezerros IA, 15 bezerros FIV e 15 bezerros clonados que foram avaliados imediatamente após o parto e com 6, 12, 24 e 48 horas de vida. Em todos os momentos experimentais foram colhidas amostras de sangue venoso para hemogasometria e foi realizado o exame clínico. Na avaliação pós-parto foram utilizados escore Apgar e mensurações de comprimento de coluna e amplitude respiratória. Os animais FIV não demonstraram alterações, assemelhando-se aos bezerros do Grupo IA. Os bezerros provenientes de CA apresentaram acidose pós-parto mais acentuada do que a acidose mista fisiológica esperada para neonatos, evidenciada pela diminuição dos valores de bicarbonato (HCO_3^-) e excesso de bases (EB) e pelo aumento de pressão parcial de dióxido de carbono (PCO_2) quando comparados aos demais grupos. Esse distúrbio pode ter refletido em valores médios menores de escore Apgar e no aumento das frequências cardíaca e respiratória. Sugere-se acompanhamento intensivo desses neonatos, com monitoramento por meio do exame clínico e hemogasométrico para diagnóstico precoce dessa condição e tratamento baseado em oxigenioterapia e reposição de bicarbonato.

TERMOS DE INDEXAÇÃO: Clínica, hemogasometria, bezerros, inseminação artificial, fertilização *in vitro,* clonagem animal, acidose respiratória, amplitude respiratória, bovinos, escore Apgar, neonatologia.

INTRODUCTION

Most of the advances in newborn animal health care in the last decades concern respiratory function. As one of the main causes of perinatal mortality in bovines, it is essential for adaptation and survival of neonates outside the uterus (Mee 2008, Bleul 2009). Animals born after *in vitro* fertilization (IVF) and animal cloning (AC), alongside premature neonates, are more likely to present respiratory conditions. Birgel Junior et al. (2011) observed a high incidence of neonatal asphyxia leading to death in cloned calves. Thus, proper respiratory adaptation resulting in efficient body oxygenation and good vitality is a crucial factor for the survival of these newborns.

In order to evaluate this process, blood gas analysis shows useful data on acid-base balance, which supports diagnosis and prognostics of several diseases affecting cattle (Sucupira & Ortolani 2003). In neonates, after cutting the umbilical cord and during the transition from placental to pulmonary oxygenation, minor transient respiratory and metabolic acidosis is observable due to the anaerobic glycolysis in low-perfused tissues (Vaala & House 2006). This condition is aggravated by neonates' reduced breathing capacity, characterized by hypoventilation, which hinders the efficiency of carbon dioxide (CO_2) removal, increases the production of carbonic acid, and consequently reduces blood pH (Benesi 1993, Piccione et al. 2006). To fight acid-base imbalance, the organism usually adopts the following mechanisms: chemical buffering with bicarbonate, ventilation adjustment, and renal excretion of ions. The buffer and respiratory systems act within a few minutes, while the kidneys are slow to respond to acid or base excess (Houpt 2006).

Venous blood is commonly used for blood gas analysis, as it is easier to collect than arterial blood and provides reliable results in cases of metabolic acidosis (Dibartola 1992). Sucupira & Ortolani (2003) found a high correlation among the pH, bicarbonate (HCO_3^-), and base excess (BE) values between venous and arterial blood in cattle, which indicates that venous blood may accurately reflect acid-base balance.

Clinical examination is also key to evaluate newborns in addition to the blood gas analysis (Feitosa & Benesi 2014). Following birth, the Apgar score evaluates head movement, reflexes, type of respiration, and mucosal color to identify newborns with vitality changes (Born 1981). Additionally, vital signs such as heart (HR) and respiratory rate (RR) and rectal temperature (RT) must be measured (Feitosa & Benesi 2014). The breath amplitude (BA) measure, which reflects rib cage expansion, may provide useful information on the calves' pulmonary compliance and ability to effectively ventilate. Furthermore, the size of the newborn may help or hinder birth, besides indicating fetal maturity, which makes spine length (SL) another useful measure to complement neonatal evaluation (Dantas 2015).

Given the above, this work aimed to study calves conceived by artificial insemination (AI), a more simple and common technique with regard to blood gas analysis and clinical examination, comparing them to newborns conceived by IVF and AC to evaluate if the latter are more prone to acid-base balance changes, which translates into worse neonatal respiratory adaptation. These cases require clinical intervention to ensure the animals' health and reduce high mortality rates associated with this category of newborns.

MATERIALS AND METHODS

Animals. A total of 50 neonates was used in this study: 20 calves from artificial insemination (Group AI, all male), 15 calves from *in vitro* fertilization (Group IVF, 11 females and four males), and 15 cloned calves (AC Group, 10 females and five males). The animals in the AI Group were obtained from a farm located in Descalvado, state of São Paulo; the IVF Group, from a farm in Santa Rita de Caldas, state of Minas Gerais; and the AC Group, from a commercial enterprise located in Mogi-Mirim, São Paulo. As to their breed, the neonates in the AI and IVF groups were black and white Holsteins (BWH) born by vaginal delivery, while the AC Group was composed of five BWH, five Gir, and five Nelore calves born by C-section. The deliveries, which took place in calving pens or paddocks, were followed-up. The animals were evaluated as to health and maturity, and those presenting severe health conditions or requiring treatment were excluded. All of the cows were kept in a semi-intensive regime, with access to feeding trough containing corn silage and to pasture, in addition to water *ad libitum* and the concentrated feed used in each farm. The study included cows with body condition scores between three and four (Feitosa & Benesi 2014); unhealthy calves were excluded. After birth, the calves were separated from their mothers and bottle-fed, having received their colostrum until 48 hours after birth. The experiment was carried out from July 2015 to December 2016.

Ethical aspects. This study has been approved by the Animal Research Ethics Committee (CEUA) of "Faculdade de Medicina Veterinária e Zootecnia" (FMVZ) of the "Universidade Estadual Paulista" (Unesp), Botucatu campus, under protocol number 197/2014.

Clinical and blood gas analyses. All calves were followed-up from birth to 48 hours after birth. At one minute postpartum (PP), the neonate Apgar score was measured according to a scale modified by Born (1981). Within the first PP time points, measurements were also taken, with the aid of a measuring tape, of the spine length - from the atlanto-occipital joint to the first coccygeal vertebra - and breath amplitude reflecting the rib cage expansion of the neonates. These measurements were taken while the animals were still in decubitus position, and the chest circumference was obtained from the thirteenth rib at the end of inspiration, i.e. at maximum expansion. Within the first five minutes of life, the heart and respiratory rate and rectal temperature were also evaluated in accordance with the semiological methods recommended by Feitosa & Benesi (2014). This physical examination was repeated at all experimental time points, namely: at birth, 6, 12, 24, and 48 hours PP, when venous blood was also collected for blood gas analysis by means of puncturing the jugular vein of the neonates with a 30X10 needle coupled to a heparinized 1mL syringe. The sample for blood gas analysis was processed immediately after collection in a portable pH, electrolyte, and blood gas analyzer (I-STAT®, Abbott Laboratories, Illinois, USA). CG7+ cartridges (I-STAT®, Abbott Laboratories, Illinois, USA) were used to measure pH, partial pressures of oxygen and carbon dioxide (PPO_2 e $PPCO_2$), oxygen saturation (SO_2), bicarbonate (HCO_3), total carbon dioxide (TCO_2), and base excess (BE) with temperature correction.

Statistical analysis. A One-Way Repeated Measures Anova was carried out for comparison of the evaluation times individually within each group. An analysis of variance was also carried out at each time to compare the study groups ($P<0.05$). When significance was found ($P<0.05$), the Tukey Test was applied for comparison of means ($P<0.05$). A non-parametric analysis was chosen only for the BE data, which did not present Gaussian distribution. The Friedman test (Repeated Measures) was carried out for comparison of the evaluation times individually within each group. When significance was found ($P<0.05$), the Tukey Test was applied for comparison of medians ($P<0.05$). The Kruskal-Wallis test was also carried out at each time point to compare the study groups ($P<0.05$).

RESULTS AND DISCUSSION

Tables 1 to 11 present the results obtained from clinical examination and blood gas analysis of the calves in the different groups.

The first analysis, carried out immediately after birth, was the Apgar score (Table 1), which aimed at determining the animals' vitality. All of the 50 neonates evaluated in this study obtained scores of 7/8 or 8/8, high scores indicating good vitality at birth. This is due to the fact that calves presenting severe health conditions or malformations were excluded from the study, since they did not live for the entire period of 48 hours or required treatment, which would compromise the evaluations. However, it must be noted that the AC Group obtained the lowest mean of Apgar scores, which indicates less vitality at birth compared to the two other groups.

Table 1. Apgar score, breath amplitude (BA), and spine length (SL) of the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC) at birth (0-hour time point)

Groups	0-hour time point		
	Apgar	BA (cm)	SL (cm)
AI	7.9 ± 0.3 a	73.2 ± 5.5 b	88.0 ± 8.0 a
IVF	7.7 ± 0.4 a	81.7 ± 3.6 a	92.4 ± 4.9 a
AC	7.3 ± 0.6 b	82.1 ± 1.9 a	91.1 ± 3.7 a
P	0.001	<0.001	0.099

Data presented as mean ± standard deviation. [a,b] Means followed by the same lower case letter in a column do not differ significantly by the Tukey test ($P>0.05$).

We did not observe differences in size measured by spine length (SL) among the calves (Table 1). Although the literature refers to macrosomia as a common change in cloned animals (Birgel Junior et al. 2011), this study did not identify the syndrome. This may be because the AC Group was composed by neonates from three different breeds, which could dilute variations in SL. The breath amplitude (BA) of the AI calves was lower than the IVF and cloned calves. This may be related to three situations: the animals in the AI Group presented lower breath amplitude with lower lung compliance, which would not be compatible with their clinics, as they did not present signs of neonatal asphyxia; the newborns in the AI Group simply had chests slimmer than the other calves, which naturally presented bigger circumferences for being larger; the IVF and AC groups presented higher BA values due to increased depth and difficult breathing (hyperpnea) as an attempt to compensate for deficient oxygenation. These two last factors would corroborate the tendency of cloned and IVF calves (Pimenta-Oliveira et al. 2011), as they are bigger than AI calves, to present less efficient respiratory adaptation.

The remaining vital signs, HR and RR (Tables 2 and 3) were within the physiological ranges established for neonate calves, with variations (Lisboa et al. 2003, Gasparelli 2007). Nevertheless, a few differences were noted among the groups. The calves in all three groups presented similar HR values at birth, which tended to drop afterwards, whereas differences were found among the groups throughout the evaluation period, at 48 hours, the CA Group reached the highest average of all. It is worth noting that, during the neonatal period, neurological control of the cardiovascular system is partial, since the myocardial sympathetic nerve function is incomplete. Thus, HR is variable when the animals are under stress caused, for instance, by manipulation (Feitosa & Benesi 2014). This factor may explain the increased HR in clones, since they were bred in a commercial enterprise with high economic value, they were more closely monitored than the calves in the other groups. In addition, increased HR may be a compensation for low oxygenation caused by neonatal respiratory maladaptation. The RR of the AC Group was also higher at zero, six, and 48 hours, being constantly higher than the other groups. Higher mean RR values are associated with incomplete pulmonary function, which is attributed to a compensation mechanism. High HR means associated to high RR may suggest that the respiratory adaptation of the neonates in Group AC was less efficient (i.e. slower). Therefore, these animals presented high RR as an attempt to revert hypoxia. However, in order to better analyze this hypothesis, the results of the blood gas analyses must be taken into account (Benesi 1993).

There was no difference in rectal temperature (Table 4) among the groups at the first experimental time point. At six hours, Group AI presented lower means, even lower than reference values (Feitosa & Benesi 2014), indicating a tendency for hypothermia. This may be explained by the inability of

Table 2. Heart rate (HR) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different time points of the study (0, 6, 12, 24, and 48 hours after birth)

Time points	HR (bpm)			P
	AI	IVF	AC	
T0	152.6 ± 35.2 aA	151.0 ± 22.0 aA	140.0 ± 13.0 abA	0.377
T6	152.0 ± 29.8 aA	135.2 ± 11.1 aAB	129.7 ± 14.9 bB	0.009
T12	145.1 ± 20.0 abA	132.4 ± 31.2 aA	149.0 ± 17.0 aA	0.129
T24	133.8 ± 27.2 cA	134.5 ± 14.5 aA	142.0 ± 8.6 abA	0.428
T48	133.8 ± 19.6 cB	133.9 ± 16.2 aB	149.0 ± 17.2 aA	0.031
P	0.020	0.108	<0.001	

Data presented as mean ± standard deviation. [a,b,c,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 3. Means and standard deviations for respiratory rate (RR) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	RR (bpm)			P
	AI	IVF	AC	
T0	58.4 ± 14.8 aB	57.0 ± 10.0 abB	73.0 ± 11.7 aA	0.001
T6	50.6 ± 15.3 aB	47.1 ± 11.1 bB	62.0 ± 9.5 abA	0.005
T12	59.4 ± 14.4 aA	49.7 ± 10.7 bA	57.7 ± 14.4 bA	0.097
T24	58.6 ± 19.2 aA	58.4 ± 20.8 abA	56.7 ± 10.3 bA	0.947
T48	57.6 ± 15.5 aB	64.5 ± 19.4 aAB	67.30 ± 7.6 aA	0.016
P	0.141	0.007	<0.001	

[a,b,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 4. Means and standard deviations for rectal temperature (RT) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	RT (°C)			P
	AI	IVF	AC	
T0	38.2 ± 1.2 aA	38.9 ± 0.5 abA	38.8 ± 0.8 aA	0.063
T6	37.8 ± 0.6 aB	38.6 ± 0.2 bA	38.4 ± 0.7 abA	<0.001
T12	38.2 ± 0.7 aB	38.6 ± 0.4 bA	38.2 ± 0.4 bAB	0.036
T24	38.3 ± 0.5 aA	38.7 ± 0.3 bA	38.5 ± 0.6 abA	0.111
T48	38.4 ± 0.8 aB	39.1 ± 0.5 aA	38.8 ± 0.5 aAB	0.006
P	0.123	0.005	0.013	

[a,b,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

neonates to maintain their body temperature under adverse conditions, once this experimental stage took place in wintertime and in a geographical region with low temperature means. Over time, their temperatures normalized; at 24 hours, the means of the three groups were similar.

Lisboa et al. (2002) carried out a study evaluating the effect of age on the acid-base balance of healthy female calves during their first month of life. For such, the authors used venous blood specimens of 300 female BWH calves distributed among several age groups. These groups included neonates whose blood was collected up to eight hours PP (corresponding to the zero and six-hours time points of the present study), between eight and 16 hours PP (including the 12-hours time point), between 16 and 24 hours PP (corresponding to the 24-hours time point), at two days of age (corresponding to the 48-hours time point), among other age ranges. Thus, our discussion is based on the references of the aforementioned work for most of the blood gas test values.

Blood pH values (Table 5) were similar among the groups in the zero and six-hours time points; however, compared to the pH values observed in other studies, these presented a tendency towards acidosis (Benesi 1993). This may have occurred because, in the work of Lisboa et al. (2002), the animals evaluated presented a longer life time interval (zero to eight hours), which could allow, in healthy animals, for a compensation of acidosis. It must be noted that the birth causes changes to the blood flow in the uterus and placenta, promoting physiological acidosis with blood pH values around 7.2 (Benesi 1993, Ravary-Plumioën 2009), similar to that found in the present study. At 24 and 48 hours, the IVF Group showed higher pH values, which shows a faster corrective response to physiological acidosis. At 48 hours, all three groups presented similar pH values. It is noteworthy that all groups presented increasing pH values throughout the study, indicating maturing of the respiratory system and the action of the buffer system in the neonates' organism, in addition to the increase of compensatory HR and RR to maintain homeostasis.

On the other hand, the $PPCO_2$ (Table 6) was higher than the values found in other studies for the three groups at the initial time points. This is probably due to the same reason presented above: the age range composing the initial group of Lisboa et al. (2002) ranges from the time of birth to eight hours. Thus, the animals had had more time to stabilize breathing and compensate for the physiological acidosis compared to the values found in specimens collected immediately after birth. With regard to the $PPCO_2$, no difference was observed among the groups, except at 48 hours, when the parameter was higher in the AI Group, although it remained within the reference limits. The higher $PPCO_2$ in the AI Group, associated with the faster correction of pH in the IVF Group, detailed above, can be explained by the management of the farm housing the animals, since the birth of the IVF calves were prematurely assisted with a view to reduce mortality due to difficult deliveries in order to preserve their economic value. That is, even births without dystocia were assisted, which increased their speed. Consequently, the repercussions in the acid-base balance of the animals were milder compared to the calves in the AI Group. In all groups, the $PPCO_2$ values decreased over time, showing respiratory adaptation of the neonates.

The variables HCO_3 (Table 7) and TCO_2 (Table 8) behaved similarly, in general, as bicarbonate is usually determined by the TCO_2 and represents its value, i.e. it is equal to approximately 95% of the latter total (Carlson & Bruss 2008). The values at birth were similar among the three groups, while the CA Group presented lower values at 12, 24, and 48 hours. In addition, the means of these parameters were below the values physiologically established until 12 hours in the AI and IVF groups and did not reach the expected values with compensation in the AC Group. Therefore, it is important to note the significance of the metabolic component in determining acidosis in cloned calves compared to the animals in the other study groups. In turn, the values established for the BE variable (Table 9) in neonate bovines are -1.5mmol/L for the lower threshold and about 8mmol/L for the upper threshold (Lisboa et al. 2002). At zero hour, the three study groups presented medians below

Table 5. Means and standard deviations for venous blood pH (pH) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	pH			P
	AI	IVF	AC	
T0	7.18 ± 0.12 cA	7.20 ± 0.08 cA	7.20 ± 0.06 cA	0.684
T6	7.27 ± 0.07 bA	7.31 ± 0.05 bA	7.28 ± 0.04 abA	0.131
T12	7.30 ± 0.07 abAB	7.34 ± 0.02 abA	7.26 ± 0.07 bB	0.002
T24	7.34 ± 0.06 abB	7.40 ± 0.06 aA	7.28 ± 0.09 abB	<0.001
T48	7.35 ± 0.06 aA	7.39 ± 0.03 aA	7.34 ± 0.08 aA	0.061
P	<0.001	<0.001	<0.001	

[a,b,c,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 6. Means and standard deviations for partial pressure of carbon dioxide ($PPCO_2$) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	$PPCO_2$ (mmHg)			P
	AI	IVF	AC	
T0	62.3 ± 9.9 aA	60.6 ± 6.2 aA	60.6 ± 8.3 aA	0.785
T6	53.5 ± 10.4 bA	53.5 ± 6.2 bA	52.2 ± 5.7 bA	0.880
T12	53.3 ± 10.8 bA	51.7 ± 5.2 bcA	50.0 ± 5.6 bcA	0.499
T24	51.0 ± 7.9 bA	46.3 ± 5.8 dA	49.4 ± 12.1 bcA	0.304
T48	53.3 ± 8.0 bA	47.8 ± 3.5 cdB	46.1 ± 5.7 cB	0.003
P	<0.001	<0.001	<0.001	

[a,b,c,d,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 7. Means and standard deviations for bicarbonate (HCO_3) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	HCO_3 (mmol/L)			P
	AI	IVF	AC	
T0	22.9 ± 4.5 cA	23.6 ± 3.9 bA	22.9 ± 2.9 aA	0.845
T6	24.4 ± 2.8 bcAB	26.6 ± 3.6 aA	23.9 ± 2.3 aB	0.033
T12	25.5 ± 2.7 bA	27.7 ± 3.3 aA	22.4 ± 3.7 aB	<0.001
T24	26.7 ± 3.2 abA	28.0 ± 1.5 aA	22.6 ± 2.6 aB	<0.001
T48	29.0 ± 3.4 aA	28.4 ± 1.8 aA	24.4 ± 3.3 aB	<0.001
P	<0.001	<0.001	0.235	

[a,b,c,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

-1.5mmol/L. The values increased over the PN time; the AI and IVF groups reached the normal range at six hours, while the AC Group presented values under the lower threshold until 48 hours PN, in different ways. Thus, it was once again evident that the AC Group presented insufficient alkaline reserve and higher acidosis than the other groups.

As for SO_2 (Table 10) and PO_2 (Table 11) values, we observed no differences among the groups seeing that, overall, the changes among groups over the study time would be more faithfully shown in arterial blood samples. The venous blood results did not allow clearer results as they cannot effectively reflect physiological variations (Houpt 2006).

Table 8. Means and standard deviations for total carbon dioxide (TCO_2) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	TCO_2 (mmol/L)			P
	AI	IVF	AC	
T0	24.4 ± 4.4 dA	25.3 ± 4.0 bA	24.5 ± 3.0 aA	0.779
T6	25.6 ± 3.2 cdA	28.0 ± 3.8 abA	25.4 ± 2.4 aA	0.053
T12	27.0 ± 2.7 bcA	28.9 ± 3.6 aA	23.7 ± 3.9 aB	<0.001
T24	28.0 ± 3.3 bA	29.4 ± 1.5 aA	24.2 ± 2.8 aB	<0.001
T48	30.5 ± 3.7 aA	29.6 ± 2.1 aA	25.6 ± 3.2 aB	<0.001
P	<0.001	<0.001	0.337	

[a,b,c,d,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 9. Medians, first and third percentiles obtained for base excess (BE) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	BE (mmol/L)			P
	AI	IVF	AC	
T0	-5.0 (-9.0 – -1.5) cA	-3.0 (-5.0 – -1.0) bA	-6.0 (-6.8 – -1.8) aA	0.466
T6	-3.0 (-4.0 – 0.0) cA	-1.0 (-2.0 – 4.5) abA	-3.0 (-4.8 – -1.0) aA	0.053
T12	0.0 (-2.5 – 1.5) bcAB	2.0 (0.0 – 2.0) aA	-5.0 (-6.0 – 0.0) aB	<0.001
T24	2.0 (0.0 – 4.0) abA	4.0 (3.0 – 4.8) aA	-5.0 (-6.5 – -2.0 aB	<0.001
T48	3.5 (2.0 – 7.0) aA	4.0 (2.0 – 6.0) aA	-2.0 (-4.8 – 4.0) aB	0.004
P	<0.001	<0.001	0.240	

[a,b,c,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 10. Means and standard deviations for oxygen saturation (SO_2) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	SO_2 (%)			P
	AI	IVF	AC	
T0	25.8 ± 11.8 bA	24.0 ± 6.7 cA	20.7 ± 4.0 bA	0.234
T6	31.3 ± 10.1 abA	27.7 ± 10.1 bcA	25.5 ± 7.3 abA	0.193
T12	32.2 ± 12.2 abA	31.0 ± 8.8 bcA	28.4 ± 11.7 aA	0.599
T24	36.2 ± 12.3 aA	38.9 ± 13.9 abA	28.0 ± 11.3 abA	0.053
T48	33.3 ± 10.0 abB	42.9 ± 13.8 aA	27.3 ± 5.6 abB	<0.001
P	0.008	<0.001	0.040	

[a,b,c,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

Table 11. Means and standard deviations for partial pressure of oxygen (PPO_2) in the groups of calves conceived by artificial insemination (AI), *in vitro* fertilization (IVF), and animal cloning (AC), at the different test time points (0, 6, 12, 24, and 48 hours after birth)

Time points	PPO_2 (mmHg)			P
	AI	IVF	AC	
T0	23.95 ± 6.28 aA	23.07 ± 3.26 bA	21.60 ± 2.69 aA	0.336
T6	23.65 ± 4.65 aA	22.33 ± 3.90 bA	21.47 ± 4.02 aA	0.319
T12	23.70 ± 4.54 aA	22.93 ± 3.60 bA	22.73 ± 5.39 aA	0.801
T24	25.15 ± 5.20 aA	24.87 ± 5.08 abA	22.47 ± 6.77 aA	0.349
T48	23.35 ± 4.20 aB	28.13 ±5.90 aA	21.33 ± 3.44 aB	<0.001
P	0.645	0.004	0.799	

[a,b,A,B] Means followed by the same lower case letter in a column and upper case in a row do not differ significantly by the Tukey test (P>0.05).

CONCLUSIONS

We conclude that, particularly in cloned calves, PP acidosis was more accentuated than the physiological acidosis expected for neonates, especially with regard to the metabolic component notable in the HCO_3^-, TCO_2, and BE changes compared to the other groups. This imbalance reflected in a lower Apgar score and higher heart and respiratory rate in the AC Group.

The animals conceived by IVF did not present changes beyond the expected for newborn animals, similar to the ones presented by the AI Group. Therefore, this study confirms the need for intensive follow-up of newborn clones to allow for early diagnosis and proper treatment of this condition, seeing that it took them over 24 hours to overcome the physiological acidosis expected for bovine neonates.

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

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Original Article
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Canine lymphomas diagnosed in southern Brazil from 2000 to 2017: epidemiology and immunophenotype[1]

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ABSTRACT.- Coelho A.C.B., Echenique J.V.Z., Lemos B.S., Estima-Silva P., Sallis E.S.V., Raffi M.B. & Schild A.L. 2019. **Canine lymphomas diagnosed in the southern Brazil from 2000 to 2017: epidemiology and immunophenotype.** *Pesquisa Veterinária Brasileira 39(7):492-498.* Laboratório Regional de Diagnóstico, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário s/n, Centro, Pelotas, RS 96010-900, Brazil. E-mail: alschild@terra.com.br

Lymphoma is a neoplasm that originates from solid hematopoietic tissues and is one of the most common tumors in dogs. The goal of the present study was to perform a retrospective study of canine lymphomas diagnosed at the "Laboratório Regional de Diagnóstico", at the "Faculdade de Veterinária" of the "Universidade Federal de Pelotas" (LRD-UFPel) from 2000 to 2017, to determine the epidemiology and anatomical distribution, and to evaluate the histopathological and immunohistochemical aspects of each case according to the adapted Kiel classification. The protocols for necropsies and biopsies in the laboratory were reviewed. Lymphoma was diagnosed in 77 dogs. Approximately 37.7% (29/77) of affected dogs had no defined breed, while dogs with defined breeds accounted for 58.4% (45/77) of the diagnoses. The occurrence in males (40/77) was slightly higher than that in females (36/77), and the mean age was 8.1 years (1.4-17 years). The most affected age group was between six and 10 years of age with 31 cases (40.2%). Regarding the anatomical classification, the multicentric form was the most prevalent, accounting for 71.4% (55/77) of the diagnoses. In 40 cases that immunophenotyping was performed, B-cell lymphomas represented 62.5% of the diagnoses (25/40), while T-cell lymphomas corresponded to 37.5% of the diagnoses (15/40). The degree of malignancy according to the modified Kiel classification was low in 35% of lymphomas (14/40) and high in 65% of cases (26/40). The multicentric form was more frequent in the region of influence of the LRD-UFPel. Identification of the immunophenotype can improve the quality of life and survival in affected dogs since it allows the most appropriate treatment for each patient.

INDEX TERMS: Canine, lymphomas, diagnosis, southern Brazil, epidemiology, immunophenotype, immunophenotyping.

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RESUMO.- [**Linfomas caninos diagnosticados na região Sul do Rio Grande do Sul de 2000 a 2017: epidemiologia e imunofenótipo.**] O linfoma é uma neoplasia com origem nos tecidos hematopoiéticos sólidos e é um dos tumores mais frequentes em cães. O objetivo do presente trabalho foi efetuar um estudo retrospectivo dos linfomas caninos recebidos no Laboratório Regional de Diagnóstico, da Faculdade de Veterinária da Universidade Federal de Pelotas (LRD-UFPel) de 2000 a 2017, determinando a epidemiologia e a distribuição anatômica, bem como os aspectos histopatológicos e imuno-histoquímicos de

cada caso de acordo com a classificação de Kiel adaptada. Foram revisados os protocolos de necropsias e biópsias recebidos no laboratório identificando-se 77 casos de cães com diagnóstico de linfoma. A doença afetou cães sem raça definida em 37,7% (29/77) dos casos, enquanto os cães com raças definidas tiveram 58,4% (45/77) dos diagnósticos. A ocorrência em machos (40/77) foi discretamente maior do que em fêmeas (36/77) e a idade média foi de 8,1 anos (1,4-17 anos). A faixa etária mais acometida foi entre seis e 10 anos de idade com 31 casos (40,2%). Quanto à classificação anatômica a forma multicêntrica foi a mais prevalente atingindo 71,4% (55/77) dos diagnósticos. Em 40 casos em que a imunofenotipagem foi realizada, os linfomas de células B representaram 62,5% dos casos (25/40), enquanto os linfomas de células T equivaleram a 37,5% dos diagnósticos (15/40). O grau de malignidade de acordo com a classificação de Kiel modificada foi baixo em 35% dos linfomas (14/40) e alto em 65% dos casos (26/40). Conclui-se que a forma multicêntrica é mais frequente na região de influência do LRD-UFPel e que a identificação do imunofenótipo pode melhorar a qualidade de vida e dar maior sobrevida aos cães afetados uma vez que permite o tratamento mais adequado para cada caso.

TERMOS DE INDEXAÇÃO: Caninos, linfoma, diagnóstico, Rio Grande do Sul, epidemiologia, imunofenótipo.

INTRODUCTION

Lymphoma, which is also called lymphosarcoma, is a common hematopoietic neoplasm in dogs and accounts for 7% to 24% of all canine tumors and up to 83% of hematopoietic neoplasms in this species (Vail & Young 2007, Vail 2010). The etiology of lymphoma has been reported to be multifactorial and includes viral infections, genetic predisposition, and environmental factors (Cunha et al. 2011). Human non-Hodgkin's lymphoma (NHL) has several characteristics in common with canine lymphoma and because of this similarity, the schemes used for morphological classification of human NHL, such as those of the Kiel and Working Formulation, have been used with success by several authors to classify canine lymphoma (Moreno & Bracarense 2007, Suzano et al. 2010). Both systems classify lymphoma according to the degree of malignancy, and the World Health Organization (WHO) classifies lymphoma anatomically according to its origin as multicentric, mediastinal or thymic, alimentary, cutaneous and extranodal, of which multicentric lymphoma is the most common presentation (Owen 1980).

The large amount of tumor biology information in different neoplastic types in each species requires careful diagnosis and encompasses cellular and molecular characteristics that go beyond the definition of malignancy (Ramos-Vara et al. 2008). Immunohistochemistry (IHC) is an important tool for characterizing neoplastic diseases in humans and has also become important in veterinary medicine. This technique is fundamental in routine diagnostics for identifying different histological types and prognostic factors, directing appropriate treatment for the affected animals, and identifying patients with a higher risk of relapse and fatal course of disease (Capelozzi 2009). In veterinary medicine, there are few studies on the epidemiological, clinical, morphological and phenotype characteristics of lymphoma in dogs (Moreno & Bracarense 2007).

The objective of the present study was to perform a retrospective study of canine lymphomas diagnosed at the "Laboratório Regional de Diagnóstico", of the "Faculdade de Veterinária" of the "Universidade Federal de Pelotas" (LRD-UFPel) from 2000 to 2017, to determine its epidemiology and anatomical distribution, to describe the histopathological and immunohistochemical aspects of 40 diagnosed cases according to the Kiel classification, and to determine the predominant immunophenotype in dogs in the region of influence of the LRD-UFPel.

MATERIALS AND METHODS

Records from biopsies and necropsies performed from 2000 to 2017 by the LRD-UFPel were reviewed. Epidemiological information (breed, sex and age), histological descriptions and anatomical locations of the tumors were retrieved. The dogs were grouped by age, as follows: 1) up to two years, 2) from two to five years, 3) older than five years but younger than 10 years, and 4) older than 10 years (Cunha et al. 2011). Regarding the size of the breed, the dogs were divided into giant or large breeds, medium breeds and small breeds. To perform the anatomical classification, the criteria proposed by the World Health Organization (OMS) were used. In addition, 40 cases were selected and histologically reassessed and classified according to the modified Kiel system (Suzano et al. 2010) and were immunophenotypically characterized using immunohistochemical technique. For the detection of immunophenotype B, anti-CD79a antibody (CD79A Protein, Mouse, Recombinant - ECD, Fc Tag - BIOCARE MEDICAL) was used at a dilution of 1:100, and for T-immunophenotype, anti-CD3 antibody (Rabbit Monoclonal, EP41 - IgG BIOCARE MEDICAL) was used at a dilution of 1:100 in 7.2% PBS solution. Histological sections 3-µm thick were mounted on charged slides, were deparaffinized and rehydrated in a xylol and alcohol battery. For endogenous peroxidase blocking, 10% hydrogen peroxide solution diluted in methanol was added for 20 minutes at room temperature. Antigen retrieval for anti-CD79a was performed using TRIS solution after microwaving for 15 minutes, and for anti-CD3 antigen retrieval, proteinase K was used for 15 minutes at room temperature. Blocking of nonspecific reactions (background) was performed with 5% skim milk diluted in 7.2% PBS for 25 minutes. The sections were covered with the solution containing the antibody and incubated in a humid chamber for 18 hours (overnight) at 4°C. The sections were incubated with MACH 4 Mouse Probe for 15 minutes at 33°C and then incubated with MACH 4 HRPPolymer for 30 minutes at 35°C, both of which were purchased from BIOCARE MEDICAL (MACH 4 Universal HPR-Polymer Detection System). The chromogen associated detection system (DAB) was applied for approximately 40 seconds. The slides were then sheared with Harris hematoxylin for approximately 15 seconds, washed in distilled water and dehydrated in increasing alcohol gradations, clarified in xylol and mounted with Entellan. For the negative control, 7.2% nonimmune PBS was used instead of the primary antibody, and as a positive control, unchanged swine tonsils were used. The slides were evaluated under an optical microscope and classified as positive or negative.

RESULTS

From 2000 to 2017, 77 cases of canine lymphoma were diagnosed in the LRD: 38 in biopsies/organs and 39 in necropsies. Thirty-one (40.2%) dogs were 5 to 10 years-old; 18 (23.4%) were older than 10 years; 17 (22.1%) were 2 to 5 years-old; and 2 (2.6%) were aged less than 2 years.

The mean age of the affected dogs was eight years and one month. In nine protocols, the age was not reported. Regarding the sex of dogs affected by the disease, 40 cases (52%) were observed in males, and 36 cases (47%) were observed in females; in one case, the sex of the animal was not reported. Out of 77 cases, 45 occurred in dogs with a defined breed, and 29 occurred in dogs with an undefined breed. In three cases, the breed was not recorded in the protocols. Out of the 45 dogs with a defined breed, 22 (48.9%) were giant or large breeds, 14 (31.1%) were medium-sized breeds, and 9 (20%) were small breeds. Regarding the anatomic classification, the multicentric presentation was observed in 55 cases (71.4%), followed by the alimentary form in nine cases (11.7%), the cutaneous form in seven cases (9.1%), and extranodal disease in six cases (7.8%).

Out of the 40 cases of canine lymphoma selected for immunophenotyping, 21 were from necropsies, and 19 were from biopsies. The course of the disease from the first clinical signs until death or euthanasia was recorded in the protocols of 13 necropsies. Out of these 13 cases, 10 were positive for B cells, eight were multicentric, one was alimentary and one extranodal, and the course varied from three days to three years; in the dog with extranodal lymphoma, the disease duration was three days. Of the three cases that were positive for T cells, all were multicentric, and the course of disease ranged from two weeks to six months. In two biopsies that were sent to the LRD-UFPel and were both positive for B cells and multicentric, the survival of the affected dog in one case was two years and eight months, and in the other was four weeks. In the patient with the longer disease course, the dog was treated with the protocol indicated for the disease (a combination of cyclophosphamide, vincristine and prednisone for four weeks and maintenance with lomustine for five months). The patient with the duration of disease of one month was not treated.

Forty cases of lymphoma were diffuse, and the number of cases and the histological characteristics (Fig.1A-D), according to the Kiel criteria are presented in Table 1. The degree of malignancy according to the modified Kiel classification was low in 35% of lymphomas (14/40) and high in 65% of lymphomas (26/40). According to the results of immunohistochemistry, B-cell lymphomas represented 62.5% of the cases (25/40), while T-cell lymphomas corresponded to 37.5% of the cases (15/40). Table 2 shows the frequency of the cell types observed

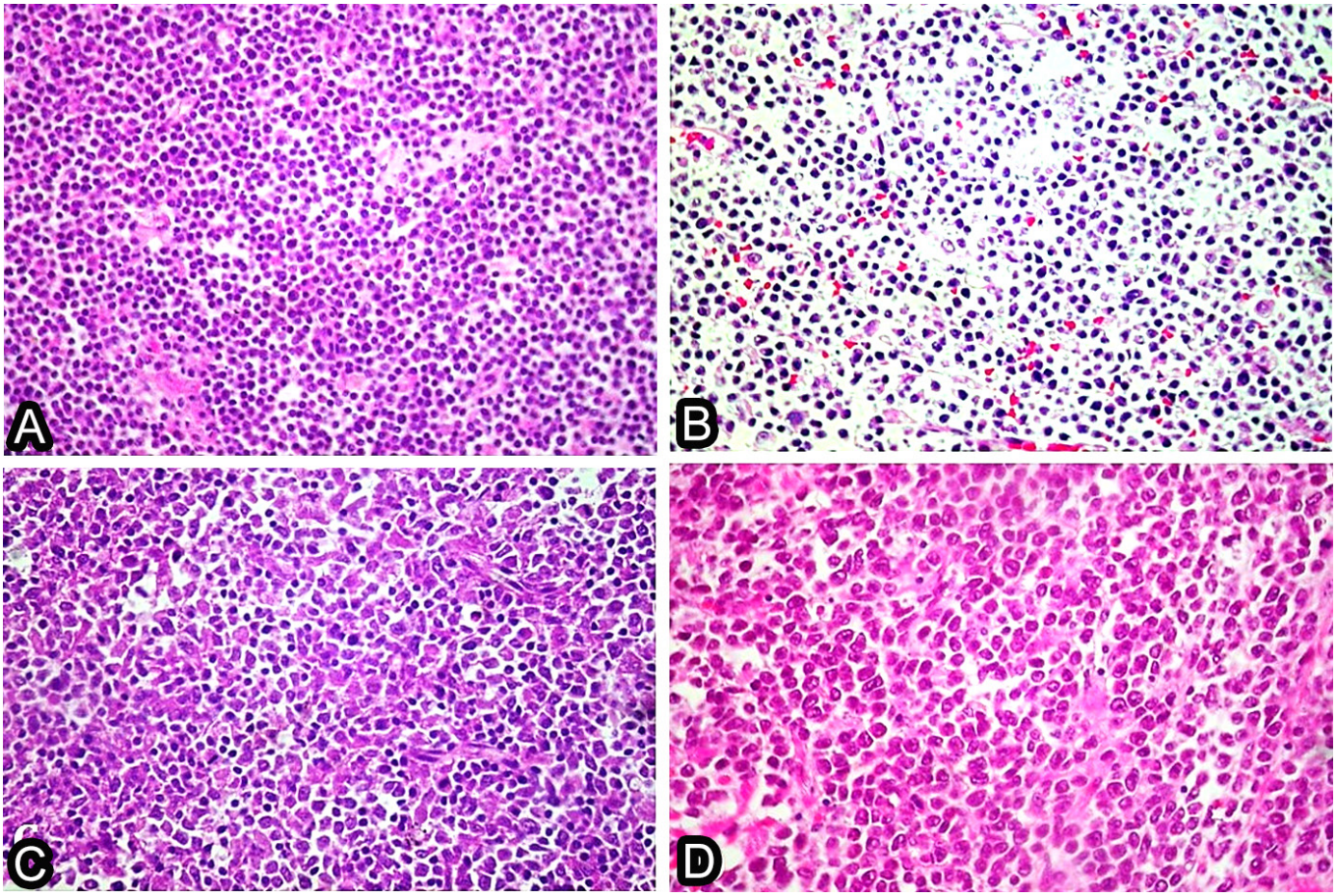


Fig.1. Lymphoma in dog. (**A**) Lymphocytic lymphoma. Small cells, with small rounded nuclei, dense chromatin and scarce cytoplasm. HE, obj.20x. (**B**) Centrocytic lymphoma. Small cells with scarce cytoplasm, with small and irregular nuclei, loose to dense chromatin. HE, obj.20x. (**C**) Centrocytic-centroblastic lymphoma. Two distinct cell groups, small cells similar to the centrocytic type and large cells similar to the centroblastic type. HE, obj.20x. (**D**) Centroblastic lymphoma. Large cells with slightly basophilic cytoplasm, ranging from scarce to abundant. Rounded nuclei, loose chromatin, with multiple medium size peripheral nucleoli, and mitoses. HE, obj.20x.

in each case of lymphoma according to the Kiel classification, as well as the immunophenotype of each tumor.

Regarding the anatomical classification of the 40 selected cases, the multicentric form occurred in 65% (26/40) of dogs with a history of generalized lymphadenopathy; 17 were diagnosed at necropsy, and nine were diagnosed by biopsies. Eighteen dogs were positive for B cells, and eight were positive for T cells. The cutaneous form occurred in 12.5% (5/40) of the dogs, and all of them were positive for T cells; the alimentary form occurred in 12.5% (5/40) of the dogs, and all of them were positive for B cells; and the extranodal form occurred in 10% (4/40) of the dogs, with two dogs being positive for B cells (Fig.1A) and two dogs being positive for T cells (Table 2, Fig.2B).

Table 1. Number of cases and histological characteristics of the lymphomas observed according to the criteria of the modified Kiel system

Modified Kiel system	Number of cases	Histological characteristics
Low grade of malignancy		
Lymphocytic	2	Small cells, with small rounded nuclei, dense chromatin and scarce cytoplasm.
Centrocitic	4	Small cells with scarce cytoplasm, with small and irregular nuclei, loose to dense chromatin, and nucleolus present or absent.
Centrocitic-centroblastic	8	Two distinct cell groups, small cells similar to the centrocytic type and large cells similar to the centroblastic type.
High degree of malignancy		
Centroblastics	9	Large cells with slightly basophilic cytoplasm, ranging from scarce to abundant. Rounded nuclei, loose chromatin, with multiple medium size peripheral nucleoli, and mitoses.
Immunoblastic	9	Cells of medium to large cytoplasm, ranging from abundant to sparse, round to oval nucleus, vesicular chromatin and prominent and central nucleolus, and the many mitotic figures.
Lymphoblastic	3	Rounded, small to medium-sized cells with scanty cytoplasm, with a clear alo around cells, rounded nuclei, dense chromatin and mitotic figures.
Anaplasic	5	Large and pleomorphic cells with slightly basophilic cytoplasm, large, rounded and irregular nuclei, loose chromatin and mitotic figures.

Fonte: Suzano et al. 2010

Table 2. Histological and immunuhistochemical classification of 40 cases of canine lymphomas, age, sex and anatomical location

Lymphoma subtypes classification	Number of cases	Average age (years)	Sex		Anatomical location			
			M	F	Mt	Al	Ct	Ex
B Cell lymphoma	25							
Low grade of malignancy	10							
Lymphocytic	1	1		1	1			
Centrocytic	2	9.5	2		2			
Centrocytic-centroblastic	7	7.9	6	1	5	2		
High degree of malignancy	15							
Centroblastic	4	7.7	2	2	4			
Immunoblastic	6	10.2	2	4	3	3		
Lymphoblastic	1	7	1					1
Anaplasic	4	7.3	1	3	3			1
T Cell lymphoma	15							
Low grade of malignancy	4							
Lymphocytic	1	6		1	1			
Centrocytic	2	8.5	1	1	1		1	
Centrocitic-centroblastic	1	9		1	1			
High degree of malignancy	11							
Centroblastic	5	7	3	2	4			1
Immunoblastic	3	7.7	1	2			3	
Lymphoblastic	2	13	1	1	1			1
Anaplasic	1	2.5		1			1	
TOTAL	40		20	20	26	5	5	4

M = male, F = female, Mt = multicentric, Al = alimentary, Ct = cutaneous, Ex = extranodal.

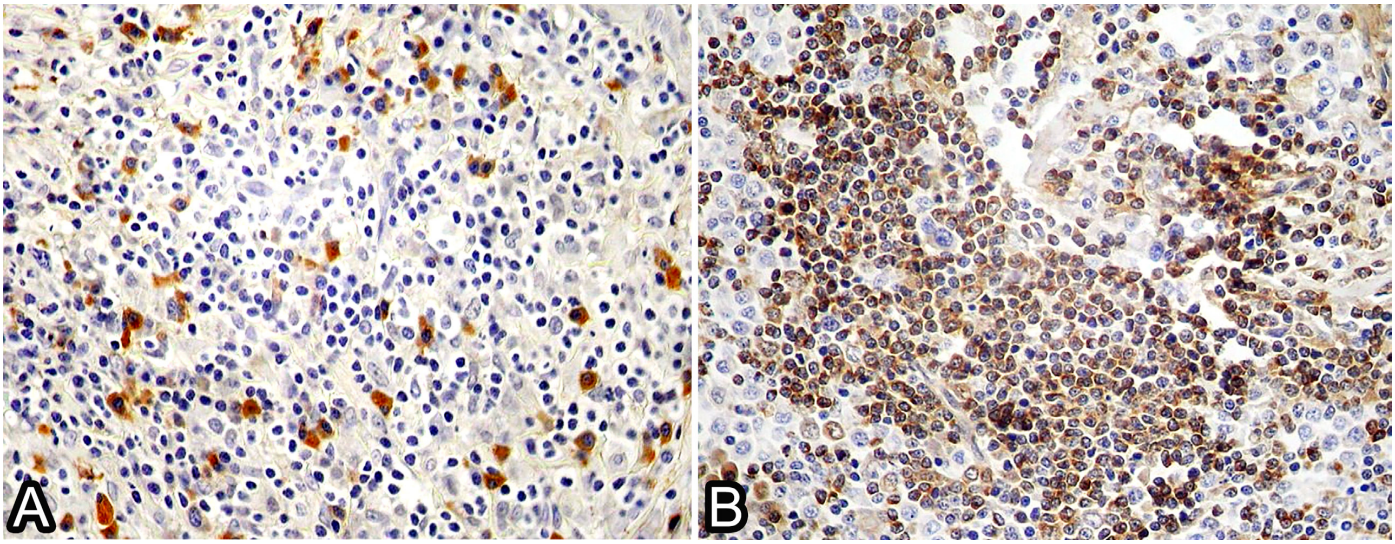


Fig.2. Lymphoma in dog. (**A**) Immunoblastic lymphoma positive for anti-CD79a antibody (B cell). IHC, obj.20x. (**B**) Centrocytic-centroblastic lymphoma positive for anti-CD3 antibody (T-cell). IHC, obj.20x.

DISCUSSION

In the present study, 29 dogs with lymphoma had no defined breed, and 48 patients were of various breeds, with Rottweilers being the most affected dog breed. In other reports, it has been shown that breeds, such as Rottweilers, Boxers, German Shepherds, Cocker Spaniels and Poodles, develop lymphoma more frequently than other dog breeds (Cardoso et al. 2003, Moreno & Bracarense 2007, Cunha et al. 2011). If we consider the individual breed, the incidence of lymphoma in dogs with an undefined breed was much higher than in those with defined breeds. It is estimated that in the municipality of Pelotas, where most of the dogs in this study came from, that there are approximately 66,700 dogs, of which approximately 80% have no defined breed or are crossbred (Prefeitura Municipal de Pelotas 2012). This could justify the large number of cases in undefined breed dogs.

It was observed that sex apparently does not interfere with the occurrence of lymphoma, which has also been mentioned by other authors (Cunha et al. 2011), although there have been reports of a predisposition to lymphoma in males (Fighera et al. 2006, Moreno & Bracarense 2007, Gavazza et al. 2009).

The mean age of dogs affected by lymphoma was eight years, and the highest prevalence of the disease occurred in middle-aged dogs. In a similar study, in the central region of Rio Grande do Sul, the authors observed a tendency to develop lymphoma in elderly animals (Fighera et al. 2006). On the other hand, in a study conducted in the North of Paraná, the age group of dogs most frequently affected by the disease was between two and five years of age (Moreno & Bracarense 2007). This age group was also the most affected in a study carried out in the United States (Teske 1994). These differences suggest that there is no age pattern for the occurrence of lymphomas in dogs; however, animals less than 24 months of age are rarely affected.

According to the anatomical classification, the multicentric form was the most frequently diagnosed, accounting for 71.4% of the cases. In other studies, it has been demonstrated that the multicentric form is the most frequent form, with percentages higher than 80% (Teske 1994, Ponce et al. 2010, Neuwald et al. 2014). The high degree of malignancy observed in most lymphomas (65%) in this study has also been observed by other authors (Teske et al. 1994, Fournel-Fleury et al. 2002, Suzano et al. 2010, Ponce et al. 2010). These findings demonstrate that the behavior of lymphoma with respect to its anatomical location and malignant potential are similar regardless of the region of the study. On the other hand, in terms of the cellular origin, there appears to be a regional or geographical influence that interferes with the cell types of canine lymphoma (Teske et al. 1994, Fournel-Fleury et al. 2002). In the present study, most of the diagnosed lymphomas were B-cell lymphomas (62.5%), and in a similar study carried out in São Paulo, the most frequent type was T-cell lymphoma, which accounted for 60.2% of cases (De Moura et al. 2001). These differences may be related to geographical differences. In phenotype studies performed in man, it has been shown that in European countries and the United States, the most frequent phenotype is the B-cell phenotype, with the T-cell phenotype occurring in only 12% of cases. In Japan, this percentage increases to 50% (Fournel-Fleury et al. 2002). Geographic differences may influence the immunophenotype of lymphomas in man, which can be extrapolated to dogs (Fournel-Fleury et al. 2002). However, in the State of São Paulo, in a study of the immunophenotypes of lymphomas in humans, a predominance of B-cell lymphoma was found (Gouveia et al. 2011), whereas in a study in dogs, also in São Paulo, T-cell lymphoma was predominant. Ponce et al. (2010) observed 35.4% of T-cell lymphomas in dogs and suggested that this percentage may be related to the greater number of skin biopsies performed, all with a T-cell immunophenotype. In the present study, out of seven skin samples with a diagnosis of lymphoma, five were submitted for immunohistochemistry to determine the immunophenotype, and all originated from T cells; however, in the LRD case, the skin samples did not outperform the other materials sent for diagnosis, which ruled out the influence of anatomical location on the percentage of T-cell lymphomas in the dogs of this study.

In the 13 cases of lymphoma in dogs that were diagnosed at necropsy, considerable variation was observed in the duration of the disease, independent of immunophenotype. During the advanced stage of the disease, regardless of the immunophenotype, the course of disease is rapid (Machicote & González 2008). In four cases of multicentric lymphoma that were positive for B cells, considering that the immunophenotype that is less aggressive, the evolution was less than one week, and in one case of multicentric T-cell lymphoma, which is more aggressive, the observed course of disease was two weeks. It should be noted that the information on the treatment of these animals was not available, and treatment should be considered since it may influence the survival time of the animals. Lymphomas, although highly heterogeneous, are considered to be responsive to chemotherapy. However, in dogs, survival of more than one month is not frequent in untreated animals (MacEwen 1990). This suggests that these five dogs were not treated for the disease. In two diagnoses made by biopsy, in which the protocol provided information on the survival time of dogs, it was found that one of the dogs that were treated with chemotherapy survived for two years, and the other dog that did not undergo chemotherapy survived for a month. In a study of 147 dogs with lymphoma, the mean survival time with complete or partial regression of increases in lymph node volume was 265 days (MacEwen et al. 1987). Most likely, the efficiency of chemotherapy is directly associated with the immunophenotype of lymphoma, which indicates that detection of immunophenotype by immunohistochemistry, is necessary considering the maintenance and quality of life of the pets affected by the disease. The extranodal form is rare in dogs (Jacobs et al. 2002), and in the present study, three of the four cases that were submitted for immunophenotyping were from biopsies and were be classified as multicentric if other organs were involved. The incidence of mediastinal lymphoma has been reported to be low in some studies (Sequeira et al. 1999, Fournel-Fleury et al. 2002) and relatively high in others (Jacobs et al. 2002, Cardoso et al. 2003). In the present study, no cases of mediastinal lymphoma were diagnosed.

The determination of the immunophenotype of lymphoma is of undeniable importance, since it allows detailed diagnosis and prognosis and directs the choice of a more appropriate treatment (Horta et al. 2012). In a study by Kiupel et al. (1999) using the same antibodies from the present study, it was demonstrated that the determination of the immunophenotype in dogs with lymphoma was important for the prognosis of survival, indicating that dogs with B-cell lymphomas, which are less aggressive, were those with a longer survival time (Teske et al. 1994). In the present study, the percentage of tumors with a T-cell immunophenotype and a high degree of malignancy based on morphology was greater (73%) than that of low malignancy (27%). Apparently, from these results, it is possible to corroborate the observations made by Teske et al. (1994), who suggested that T-cell lymphomas always have a high degree of malignancy, independent of cell morphology. In addition, cutaneous lymphoma can be considered to be T-cell lymphoma in origin, and treatments directed to this cell type should be performed when phenotyping is not possible.

CONCLUSIONS

The results of the present study demonstrated that canine lymphoma presents mostly immunophenotype B and is multicentric, considering the region of influence of the LRD-UFPel.

In cases of cutaneous lymphoma, when it is impossible to perform the phenotyping, treatment protocols for tumors originating from T cells, seems to be the most appropriate.

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

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Clinical-epidemiological, anatomic-pathological, histochemical and immunohistochemical characterization of renal cystadenocarcinoma-nodular dermatofibrosis syndrome in 11 German Shepherd dogs[1]

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ABSTRACT.- Thompson R.P.M., Lamego E.C., Melo S.M.P., Irigoyen L.F., Fighera R.A. & Kommers G. D. 2019. **Clinical-epidemiological, anatomic-pathological, histochemical and immunohistochemical characterization of renal cystadenocarcinoma-nodular dermatofibrosis syndrome in 11 German Shepherd dogs**. *Pesquisa Veterinária Brasileira 39(7):499-509.* Laboratório de Patologia Veterinária, Departamento de Patologia, Universidade Federal de Santa Maria, Av. Roraima 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: glaukommers@yahoo.com

Eleven cases of renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND) are described in German Shepherd dogs diagnosed from January 1994 to January 2018 at the Veterinary Pathology Laboratory of the "Universidade Federal de Santa Maria" (LPV-UFSM). The study sample was composed of eight male and three female dogs at a ratio of 2.67:1. Age ranged from six to 12 years (mean=8.7 years). The main clinical signs reported in descending order of frequency were multiple cutaneous nodules (nodular dermatofibrosis), dyspnea, anorexia, weight loss, recurrent hematuria, vomiting, and polydipsia. Results demonstrated that it is not always easy to clinically recognize this syndrome, but its peculiar anatomical-pathological characteristics allow safe diagnosis. Histologically, it was possible to detect all phases (cysts, papillary intratubular hyperplasia, and cystadenomas or cystadenocarcinomas) of a possible pathological *continuum* of the renal lesions. Uterine leiomyomas were observed in only one of the cases. Through histochemical techniques, it was possible to identify the presence of type I collagen in both cutaneous and renal lesions and consider its possible involvement in the pathogenesis of renal cystadenocarcinoma. Immunohistochemistry (IHC) showed partially satisfactory results in the staining of epithelial cells of renal cysts and neoplasms for pan-cytokeratin.

INDEX TERMS: Clinics, epidemiology, anatomic-pathology, histochemistry, immunohistochemistry, renal cystadenocarcinoma, nodular dermatofibrosis, syndrome, German Shepherd dogs, dog diseases, pathology.

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RESUMO.- [Caracterização clínico-epidemiológica, anátomo-patológica, histoquímica e imuno-histoquímica da síndrome cistadenocarcinoma-dermatofibrose nodular em 11 cães Pastor Alemão.] São descritos 11 casos da síndrome cistadenoma/cistadenocarcinoma-dermatofibrose nodular (CR-DN) em cães Pastor Alemão, diagnosticados entre janeiro de 1994 e janeiro de 2018 no Laboratório de Patologia Veterinária da Universidade Federal de Santa Maria

(LPV-UFSM). Os cães afetados foram oito machos e três fêmeas, estabelecendo-se uma relação de 2,67:1. A idade variou de seis a 12 anos, sendo a média de idade de 8,7 anos. Os principais sinais clínicos relatados foram, em ordem decrescente de frequência, múltiplos nódulos cutâneos (dermatofibrose nodular), dispneia, anorexia, emagrecimento, hematúria recorrente, vômito e polidipsia. Este estudo permitiu estabelecer que o reconhecimento clínico da síndrome nem sempre é fácil, porém suas características anátomo-patológicas peculiares permitem um diagnóstico com segurança. Histologicamente, foi possível detectar todas as fases (cistos, hiperplasia intratubular papilífera, cistadenomas ou cistadenocarcinomas) de um possível continuum patológico das lesões renais. Leiomiomas uterinos foram observados somente em um caso. Através das técnicas histoquímicas foi possível estabelecer que o colágeno tipo I está presente em ambas as lesões, cutâneas e renais, e cogitar seu possível envolvimento na patogênese dos cistadenocarcinomas renais. A técnica de IHQ mostrou resultados parcialmente satisfatórios na imunomarcação das células epiteliais dos cistos e dos neoplasmas renais para pancitoceratina.

TERMOS DE INDEXAÇÃO: Clínica, epidemiologia, anátomo-patologia, histoquímica, imuno-histoquímica, síndrome, cistadenocarcinoma, dermatofibrose nodular, Pastor Alemão, doenças de cães, patologia.

INTRODUCTION

Renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a rare syndrome characterized by bilateral and multifocal renal cystadenoma/cystadenocarcinoma, nodular dermatofibrosis, and uterine leiomyoma that has been reported mainly in German Shepherd dogs (Lium & Moe 1985, Moe & Lium 1997). This syndrome is caused by mutations in the folliculin gene (*FLCN*, previously *BHD*) gene, which is located in chromosome 5 and is a dominantly inherited disease (Lingaas et al. 2003, Bonsdorff et al. 2009, Pressler et al. 2009). Inactivation of this tumor suppressor gene is one of the critical steps in this disease (Bonsdorff et al. 2009). This syndrome has also been sporadically reported in Boxer, crossbred (White et al. 1998), and Golden Retriever dogs (Marks et al. 1993).

Clinical signs vary greatly among dogs depending on age and stage of the disease at examination (Moe & Lium 1997). They usually include numerous firm cutaneous and subcutaneous nodules, abdominal distension, greatly enlarged kidneys (on palpation), anorexia, fatigue, progressive weight loss, polydipsia, vomiting, and constipation or diarrhea (Lium & Moe 1985).

Gross lesions observed at necropsy are multiple firm and spherical skin and subcutaneous nodules. They may be distributed throughout the body, but with marked preference for limbs, head and back (Suter et al. 1983, Lium & Moe 1985, Moe & Lium 1997). In the kidneys, lesions are bilateral, multiple and cystic, containing a gelatinous, limpid or reddish brown fluid, often with areas of necrosis. The cysts can rupture and release their contents into the peritoneal cavity. (Lium & Moe 1985, Meuten & Meuten 2017). Affected females also tend to present several uterine leiomyomas (Cianciolo & Mohr 2016). Histologically, the cutaneous lesion is denominated nodular dermatofibrosis and, cystadenoma or cystadenocarcinoma can be observed in the kidneys (Lium & Moe 1985).

In general, most textbooks of veterinary medicine (Cianciolo & Mohr 2016, Breshears & Confer 2017, Serakides & Silva 2017) contain little, generic information on RCND, and this syndrome is mostly described in the international literature, with little information on its prevalence and presentation characteristics found in the national literature (Langohr et al. 2002, Inkelmann et al. 2012), which is important for the diagnosis and knowledge of the occurrence of this disease in the country.

The main objectives of this retrospective study were to determine the prevalence of RC-ND in the necropsies routine of the LPV-UFSM and to characterize the clinical-epidemiological, anatomical-pathological, histochemical and immunohistochemical aspects of this syndrome in German Shepherd dogs in the central region of Rio Grande do Sul state, Brazil.

MATERIALS AND METHODS

Protocols of dog necropsies conducted from January 1994 to January 2018 at the Laboratory of Veterinary Pathology of the "Universidade Federal de Santa Maria" (LPV-UFSM) were reviewed in search of cases of cutaneous and renal lesions compatible with those described for renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RC-ND) in German Shepherd dogs. The total number of German Shepherd dogs necropsied during this period was also calculated. Cases 1 to 4 and 1 to 6 of RC-ND syndrome included in this survey were part of the studies by Langohr et al. (2002) and Inkelmann et al. (2012) respectively.

From these necropsy protocols, information on the gender and age of the dogs was obtained. As for age, dogs were divided into three categories, as previously reported (Fighera et al. 2008), namely, puppies (<1 year), adults (≥1 to ≤9 years), and elderly (>10 years).

Clinical signs, outcome (spontaneous death or euthanasia), macroscopic findings (pattern of dermatofibrosis lesions, including number, size, distribution and presence of ulceration in cutaneous nodules; size, location, distribution and characteristics of renal lesions and presence of metastases), and microscopic findings were also determined. Other related injuries, such as uterine and extra-renal lesions of uremia, were also recorded.

As for gross lesions, the data were computed from the descriptions present in the medical reports, complemented by observation of the archives case photographs. Regarding histopathological evaluation, the aspects described in the necropsy reports were considered and, depending on the availability of paraffin-embedded tissues on the LPV-UFSM archives, new histological sections (stained with hematoxylin-eosin, HE) were evaluated.

Renal and cutaneous lesions, depending on the availability of paraffin-embedded tissues, were evaluated using the histochemical techniques of Masson's Trichrome (MT, Masson's Trichrome Histokit, EasyPath, EP-11-20013) and Picro-Sirius Red (PR, Picro-Sirius-Hematoxylin Histokit, EasyPath, EP-11-20011) for better collagen evidencing and typification, respectively. Using TM, the collagen areas stain in blue, whereas using PR under polarized light, type 1 collagen is observed as dense, yellow, orange or red fibers and type 3 collagen as fine, green fibers (Whittaker et al. 1994).

Renal lesions were also assessed by immunohistochemistry (IHC) using bovine polyclonal anti-pancytokeratin antibody (Dako Cytomation, code Z-0622) produced in rabbit (1:2,000). Silanized slides with 3µm histological sections were used. After dewaxing and rehydration of the tissues, antigenic recovery with TRIS-EDTA at pH 9.0 was performed, followed by endogenous peroxidase blocking with 3% hydrogen peroxide. Blocking of non-specific reactions

was performed with protein blocker (EasyPath kit, EP-12-20504). Incubation with the primary antibody occurred in oven for 60 min at 37°C in a humid chamber. Easy Link One polymer (EasyPath kit, EP-12-20504) was used as secondary antibody, incubated in oven at 25°C. 3-3'-diaminobenzidine tetrachloride (DAB; EasyPath kit, EP-12-20504) was used as substrate-chromogen. Sections were counterstained with Harris hematoxylin, dehydrated, and mounted using synthetic mounting medium (Entellan, Merck) and coverslips. Bovine alimentary tract squamous cell carcinoma was used as positive control. The same sections to be tested, with replacement of primary antibody with antibody diluent, were used as negative control.

RESULTS

Epidemiological and clinical aspects

A total of 6,231 dogs (2,958 males and 3,271 females) were necropsied during the study period. Of these, 379 were German Shepherd - 208 males (54.89%) and 171 females (45.11%). Of these, 11 (2.9%) German Shepherd dogs were diagnosed with RCND compatible lesions. The cases were chronologically distributed: two cases in 1994, 1996, and 2012; one case in 2005, 2010, 2011, 2015, and 2018. Epidemiological and clinical aspects are detailed in Table 1. Affected dogs were eight males (80%) and three females (30%), establishing a ratio of 2.67:1. Of the three bitches, two were unspayed. The age of the affected dogs ranged from 6 to 12 years. In one case, the age was not reported. Of the 10 dogs whose age was known, 7 (70%) were classified as adults and three (30%) as elderly. Overall, mean age was 8.7 years and median was 8.5 years.

The main clinical signs described in the medical reports in descending order of frequency were multiple cutaneous nodules (4/11, 36.36%), dyspnea (3/11, 27.27%), anorexia (2/11, 18.18%), progressive weight loss (2/11, 18.18%), recurrent hematuria (2/11, 18.18%), and vomiting (2/11, 18.18%). Seven dogs (7/11, 63.63%) died spontaneously and four (4/11, 36.36%) were euthanized.

Macroscopic changes

Necropsy findings usually showed enlarged kidneys and with irregular contour due to presence of firm or floating cysts and/or neoplastic nodular tissue that protruded on the capsular surface (Fig.1A,B). Table 2 shows these aspects in detail for each dog. The kidneys of the eleven dogs were bilaterally affected. In 10 cases (Dogs 1-9 and 11), cysts and neoplasms were observed in both kidneys. In one case (Dog 10), the left kidney had cysts and neoplasm, whereas the right kidney showed only small cysts.

Cut surface of the kidneys revealed cysts of a few millimeters in diameter (usually filled with clear fluid) (Fig.1C) to large cystic cavities (up to 15cm in diameter and containing clear, reddish brown and dark brown liquids, or clots) (Fig.1D), which replaced much of the renal parenchyma, affecting the cortical (mainly), medullary and pelvic (less often) regions. In most cases, the cystic cavities were also filled by scarce or abundant, dark red or dark brown, porous, extremely friable (necrotic aspect) neoplastic tissue (Fig.1E,F). Rarely (Dogs 4, 8, and 11), solid, whitish neoplastic masses were also observed. Cystic cavity capsules were generally fibrous and firm (Fig.1F). In three cases (Dogs 4, 7, and 8), there was rupture of cysts, with extravasation of contents and/or hemorrhage into the abdominal cavity (hemoperitoneum). Peritonitis was also described in Dog 8.

Skin lesions were observed at necropsy in 11 dogs. The gross characteristics of the cutaneous lesions in each case are detailed in Table 3. In nine cases, the lesions were multiple and numerous, affecting more than a single body area. A few nodules were found in one case (Dog 10) and a single nodule was observed in only one situation (Dog 1). Nodular lesions were distributed in descending order of frequency as follows: hind limbs (9/11, 81.81%) (Fig.2A), forelimbs (8/11, 72.72%), head (3/11, 27.27%) (Fig.2B), and trunk (2/11, 18.18%).

These lesions were observed as papules (<1cm) or nodules (>1cm). In several cases, nodular lesions were very

Table 1. Epidemiological and clinical aspects of 11 German Shepherd dogs with renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND)

Case	Gender	Age (years)	Clinical signs	Outcome
1	M	10	Hematuria, urinary retention, rigid and dilated urinary bladder, hind limb edema.	SD
2	M	7	Progressive weight loss (1 month), cough and dyspnea, anorexia (3 days), and vomiting (1 day).	SD
3	M	8	Numerous cutaneous nodules (2 years). Biopsy was performed three times (results not informed) and there were recurrences. No other clinical signs.	EUT
4	M	6	Polydipsia and dyspnea, died after 3 hours	SD
5	F	6	Anorexia, jaundice. Examinations: normal blood count; ALT: rose from 750 to 1195 (in 2 days). X-ray: mass in the renal region.	EUT
6	M	Adult	Cutaneous nodules on the head and limbs	SD
7	M	9	Vomiting; found in decubitus and with dyspnea, small firm nodules on the paws and head. Mass compressing the colon.	SD
8	F	9	Cystitis and hematuria (1 year). Severe weight loss and anemia. US: A mass was observed in the kidney. Anorexia. Found dead.	SD
9	M	12	Skin tumors that recurred constantly (3 years). Incoordination of the hind limbs. Developed hematuria, being diagnosed with hemorrhagic cystitis; no response to treatment.	EUT
10	M	8	Progressive weight loss, polydipsia, elevated urea and creatinine. US: fluid in the abdominal cavity and chest; changes in the left kidney and pleated intestinal segments. Exploratory celiotomy: multiple altered organs and large amount of fluid in the abdomen.	EUT
11	F	12	Found dead.	SD

M = male, SD = spontaneous death, EUT = euthanasia, F = female, ALT = alanina aminotransferase, US = ultrasound.

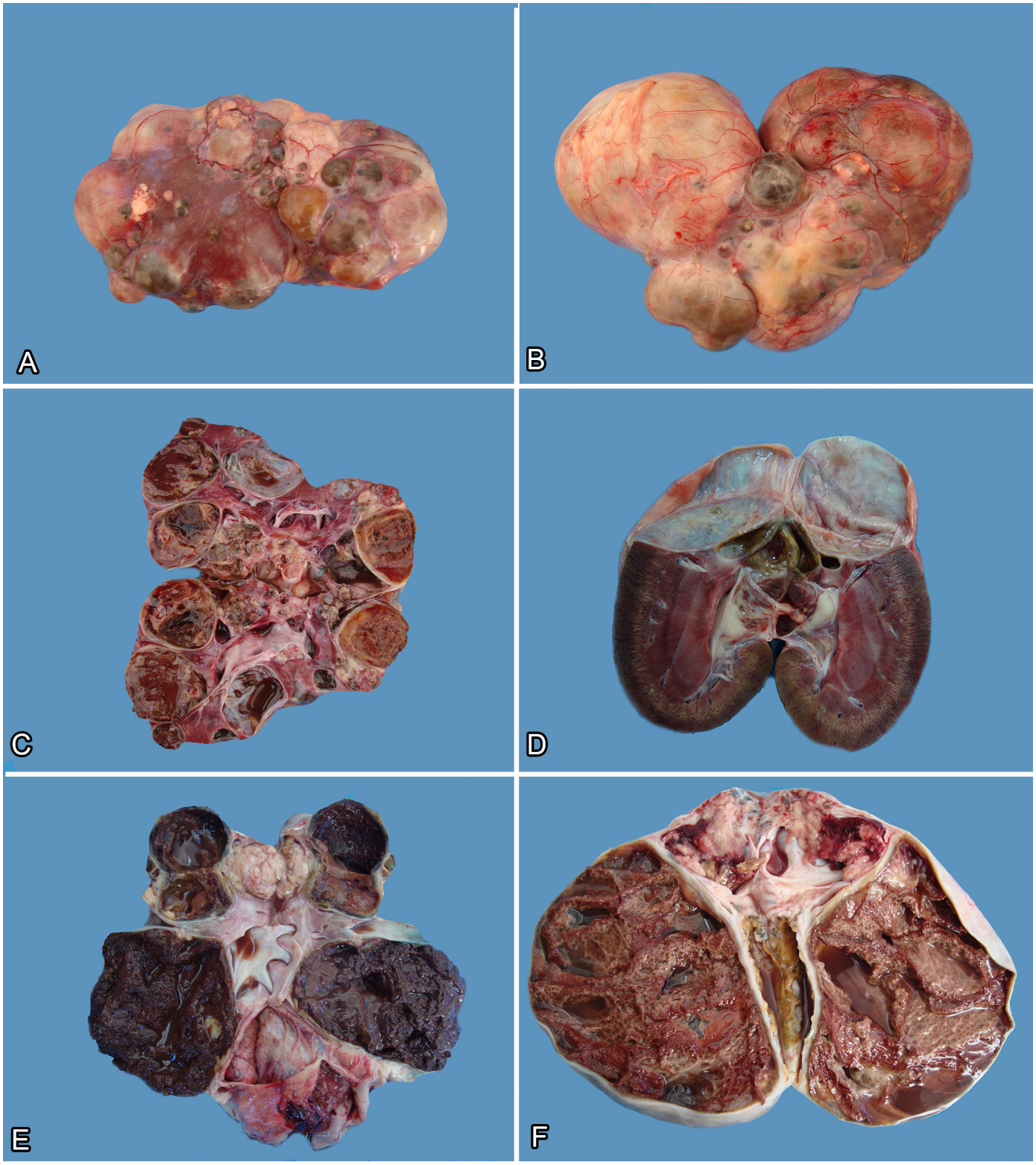


Fig.1. Renal cystadenocarcinoma. (**A**) Kidney of Dog 9 is increased in size, with parenchyma distorted by small subcapsular cystic masses. (**B**) Kidney of Dog 9 increased markedly in size with parenchyma distorted by subcapsular cystic masses of various sizes. The cysts show thick walls. (**C**) Dog 9. At cut, multiple cysts containing neoplastic and sometimes necrotic formations, are observed. There is little renal parenchyma remaining. (**D**) Dog 10. At cut, there is a cyst of approximately 3 cm in diameter located in the corticomedullary region of the cranial pole with fibrous wall and adjacent smaller cystic formations. (**E**) Dog 8. At cut, the kidney shows multiple cystic formations with dark-red, porous and extremely friable content, and some firm, whitish solid portions. (**F**) Dog 8. At cut, the kidney shows multiple cystic formations with brownish, porous and extremely friable content, and some firm, whitish solid portions. A thick, white, fibrous capsule is observed.

Table 2. Macroscopic aspects of renal lesions in 11 German Shepherd dogs with renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND)

Case	Size/weight of kidneys and cystic renal lesions	Content of cysts/cystic cavities
1	RK (30x22x7cm, with cystic cavities from 5 to 15cm in diameter) LK (with nodules of 4x6cm and 2x3cm in the poles)	Reddish-brown liquid
2	RK (16x12cm with cystic cavities) LK (19x12cm with cystic cavities)	Reddish-brown/clear liquid
3	Both (with cysts from 0.1 to 0.5cm in diameter)	Clear liquid
4	RK (with cystic cavities from 0.5 to 1.8cm in diameter) LK (with cystic cavities from 0.3 to 1.5cm in diameter)	Clot
5	RK (3-fold the normal size, with cysts from 3 to 6cm in diameter) LK (with cysts from 1 to 3cm in diameter)	Clot
6	Both (increased volume; with cysts ranging from a few mm to 3cm in diameter)	Viscous and clear liquid
7	Both (with cysts from 0.1 to 2cm in diameter)	Reddish-brown liquid/clot
8	Both (enlarged, with cystic cavities up to 4.5cm in diameter) RK (weight: 1,043g), LK (weight: 1,020g)	Reddish-brown liquid
9	Both (enlarged, with cystic cavities from 3 to 8cm in diameter)	Dark brown liquid
10	RK (with cysts up to 0.2cm in diameter) LK (enlarged, with a few cysts up to 5cm in diameter in the cranial pole)	Clear liquid
11	Both (with cystic cavities from 0.5 to 3 cm in diameter)	Reddish-brown liquid

RK = right kidney, LK = left kidney.

Table 3. Macroscopic characteristics of cutaneous lesions in 11 German Shepherd dogs with renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND)

Case	Number of nodules	Size	Site	Ulceration
1	Single	3cm in diameter	LHL	No
2	Multiple	1cm in diameter	HLs	No
3	Multiple	0.2-2cm in diameter	Head (mentum, face and periocular regions)/FLs /HLs	Yes
4	Multiple	0.3-0.6cm in diameter	RFL (ulnar region)/trunk (scapular region)	Yes
5	Multiple	0.5-1cm in diameter	LHL	No
6	Multiple	From a few mm up to 5cm in diameter	Head/FLs/HLs	No
7	Multiple	0.2-2cm in diameter	FLs/HLs/head/trunk	No
8	Multiple	0.2-0.6cm in diameter	FLs/RHL	No
9	Multiple	0.6-2cm in diameter	RFL/LHL	Yes
10	Two	4x2cm (LFL)/0.5cm in diameter (head)	LFL/head (right ear)	Yes
11	Multiple	4x2x1cm (RFL)/0.5cm in diameter (FL/HL)	FLs/HLs	No

LHL = left hind limb, HLs = hind limbs, FLs = forelimbs, RFL = right forelimb, RHL = right hind limb, LFL = left forelimb.

close to each other, sometimes coalescing and forming larger masses/plaques. The lesions were alopecic or not, sometimes ulcerated (four cases). The epidermal surface was blackened in several cases. At cut, it was observed that the nodular lesions were located in the dermis (Fig.2C) or in the subcutaneous tissue (Fig.2D), and were firm (fibrous) and white in most cases. Occasionally, soft lesions were described.

In Dog 8, there were two protruded nodules in the serosa layer of the uterus (Fig.2E), 0.3 and 0.6 cm in diameter. They were smooth and yellowish. At cut, they were not very firm and homogeneous. Additional findings associated with RCND were firm-elastic, whitish, neoplastic nodules (interpreted as metastatic) in the liver and spleen (Dog 4). In Dog 10, near the stomach (pylorus), there was a fibrous mass in the omentum, which had been transformed into a fibrous capsule that enveloped the entire intestine, causing its pleating and shrinking of the intestinal loops (Fig.2F). Small nodules were also observed in the costal pleura (and pleural effusion). The lungs showed retracted areas in the pleura and the liver had rounded edges (as a result of sharp retraction of the capsule). Extra-renal lesions of uremia were described in Dog 1 (pneumopathy) and Dog 2 (gastropathy, pneumopathy, and parathyroid hyperplasia). In the urinary bladder, urine color was described as chocolate-red (Dog 1) or dark red (Dogs 8 and 9).

Microscopic changes

Renal histological changes were multifocal, affecting the cortical and/or medullary regions, and comprised discrete epithelial cysts (Fig.3A), areas of papillary intratubular hyperplasia (Fig.3A), cystadenomas and/or cystadenocarcinomas (Fig.3B). The presence of these lesions in each case is detailed in Table 4. In the entire study sample, there were multiple small cysts composed of a thin wall and internally lined with a layer of epithelial cells, similar to the normal tubular epithelium, but generally elongated and flat (interpreted as

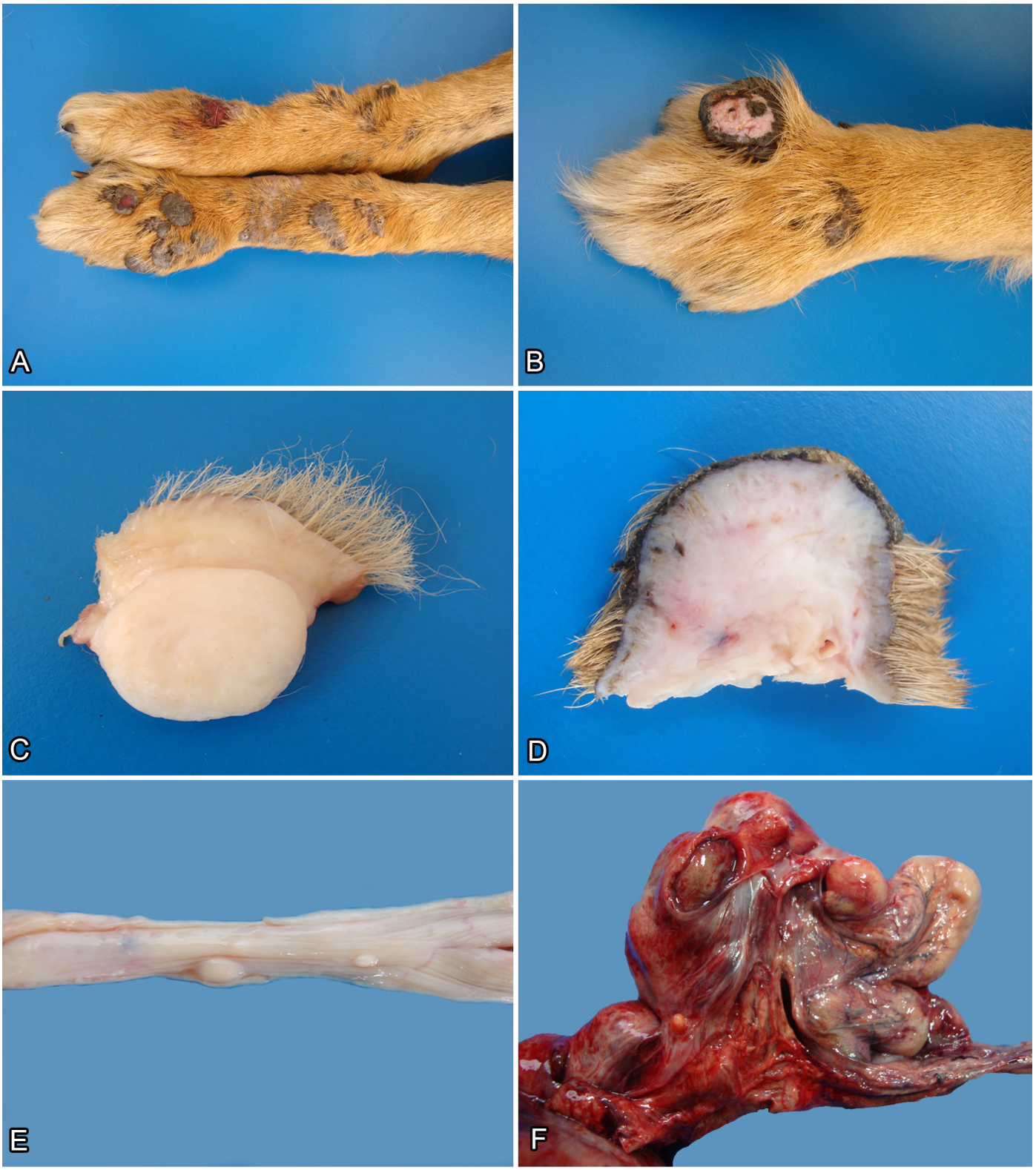


Fig.2. (**A**) Nodular dermatofibrosis in Dog 9. Skin, on the cranial face of the pelvic limbs there are multiple nodules, very close to each other, sometimes coalescing and forming larger masses, occasionally ulcerated. (**B**) Nodular dermatofibrosis in Dog 9. Multiple nodules can be observed in the cranial face of the anterior limb, with the largest nodule showing areas with ulceration and depigmentation. (**C**) Nodular dermatofibrosis in Dog 11. Skin, at cut, a white, firm nodule that extends from the superficial dermis to the deep dermis can be observed. (**D**) Nodular dermatofibrosis in Dog 9. Skin, at cut, the surface shows a firm, whitish nodule that is strictly located in the subcutaneous tissue. (**E**) Uterine leiomyoma in Dog 8. Multiple, smooth, whitish nodules can be observed in the serosa layer of the uterus. (**F**) Intestine of Dog 10. There is a mass consisting of pleated intestine, surrounded by a fibrous capsule, which corresponds to the omentum with metastasis of cystadenocarcinoma.

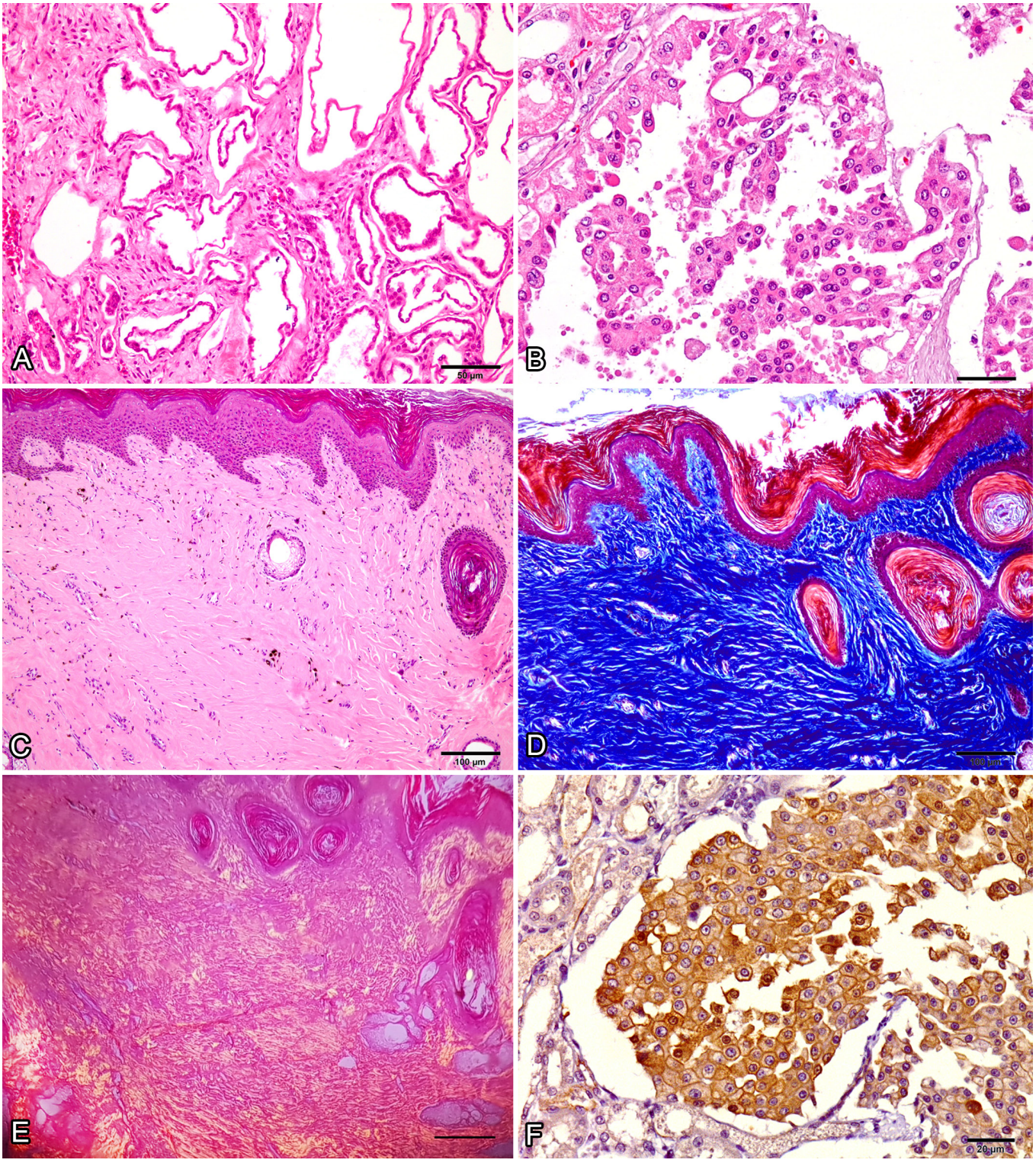


Fig.3. (**A**) Kidney. Focally extensive area with formation of cysts characterized by wall with a single layer of cells. There are two areas with onset of hyperplasia within the cysts. HE, bar=50µm. (**B**) Kidney. Proliferation of neoplastic epithelial cells forming papillary projections. There are binucleated neoplastic cells and a signet-ring cell (vacuolated cytoplasm), characteristic of renal cystadenocarcinoma. HE, bar=20µm. (**C**) Skin. Abundant presence of collagen fibers in the superficial and deep dermis (not shown in the microphotograph) and reduction of skin appendages. Irregular hyperplasia with moderate orthokeratotic hyperkeratosis is observed in the epidermis. HE, bar=100µm. (**D**) Skin. There is proliferation of collagen fibers in the superficial and deep dermis (not shown in the microphotograph) stained in blue by MT. Irregular hyperplasia of the epidermis is observed with moderate orthokeratotic hyperkeratosis and keratosis of the remaining hair follicles. MT, bar=100µm. (**E**) Skin. Same section of Figure 3D, showing thick orange collagen fibers in the dermis, characteristic of type I collagen. PR, bar=100µm. (**F**) Kidney. Positive immunostaining for pancytokeratin of the epithelial cells of a cystadenocarcinoma. IHC HRP-polymer, bar=20µm.

Table 4. Histological aspects of renal lesions in 11 German Shepherd dogs with renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND)

Case	Histological findings of the kidneys
1	Cysts, papillary intratubular hyperplasia, and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hematoidin)
2	Cysts and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hematoidin)
3	Cysts, papillary intratubular hyperplasia, and cystadenoma Peritumoral and/or intratumoral lesions: mild fibrosis
4	Cysts and cystadenocarcinoma Peritumoral and/or intratumoral lesions: mild fibrosis
5	Cysts, papillary intratubular hyperplasia, and cystadenoma Peritumoral and/or intratumoral lesions: mild fibrosis
6	Cysts, papillary intratubular hyperplasia, and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigments (hematoidin and hemosiderin)
7	Cysts, papillary intratubular hyperplasia, cystadenoma, and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigments (hematoidin and hemosiderin)
8	Cysts, papillary intratubular hyperplasia, and cystadenocarcinoma Peritumoral and/or intratumoral lesions: fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hematoidin)
9	Cysts and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hemosiderin)
10	Cysts and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hematoidin)
11	Cysts and cystadenocarcinoma Peritumoral and/or intratumoral lesions: marked fibrosis, necrosis, hemorrhage, cholesterol clefts, mineralization, and pigment (hematoidin)

caused by fluid - not stained - distension of the wall). Six cases presented some cystic formations internally lined with a high cuboidal epithelium, at times with two to three layers of cells, or forming discrete papillary projections (papillary intratubular hyperplasia). In three cases, in the wall of some cystic structures, moderate to marked papillary proliferation of well-differentiated neoplastic epithelial cells was observed, with abundant and eosinophilic cytoplasm. These cells showed single, central nuclei with a single nucleolus (cystadenomas). In general, cystadenocarcinomas were characterized as being part of a larger cystic structure that showed a thick, fibrous wall and was internally lined with neoplastic cuboidal or polyhedral epithelial cells arranged in an irregular papillary pattern. These cells presented abundant cytoplasm and round or oval, vesiculous nuclei with dispersed chromatin. Some cells were binucleated and others had a large cytoplasmic vacuole pushing the nucleus to the periphery (signet-ring cell morphology) (Fig.3B). Mitotic figures were rare. Occasionally, cystic structures filled with neoplastic cells, similar to those described for the papillary areas but arranged in a more solid pattern, were observed. The following features were frequently observed in the cystadenocarcinomas: areas of intense peritumoral (contiguous with the wall of the cystic cavities) and intratumoral (often as connective septa) fibrosis, marked multifocal necrosis sometimes containing cholesterol clefts, areas of focally extensive hemorrhage, mild multifocal mineralization, macrophage infiltrate containing golden-brown granular pigment (hemosiderin) and irregular deposits of golden-yellow crystalloid material (hematoidin).

Metastases of the cystadenocarcinomas were observed in the liver and spleen (Dog 4) and in multiple organs and tissues in Dog 10. In the latter, the fibrous mass in the omentum and the fibrous capsule into which it was transformed (which involved the entire intestine) consisted of abundant fibrous connective tissue (interpreted as a desmoplastic cirrhous reaction), well vascularized, and interspersed with moderately pleomorphic, metastatic epithelial cells, including signet-ring cells. Metastases were also observed in the esophageal serosa, costal pleura, parietal pleura, and liver capsule.

Cutaneous nodules were composed of accumulation of mature collagen, generally arranged in thick fibers and interspersed with a few fibrocytes (Fig.3C). In the dermal nodules, this collagen redundancy was observed in the skin adnexa, which were morphologically normal, or in the presence of dilated hair follicles with keratosis. In the dermis, the limits of these nodules were barely perceptible among the normal adjacent collagen. However, in the subcutaneous tissue, the collagenous nodules were well delimited among the adipose tissue. The cutaneous and subcutaneous nodules were interpreted as nodular dermatofibrosis. The epidermis was acanthotic and with orthokeratotic hyperkeratosis or ulcerated in some nodules. Mononuclear inflammatory infiltrate was occasionally observed.

In the uterus wall of Dog 8, there were well-delimited and non-encapsulated areas of neoplastic proliferation of spindle cells, which were organized in dense bundles; they were well differentiated and similar to smooth muscle cells. The cytoplasm was elongated, abundant, and eosinophilic. The nucleus was elongated and composed of moderately aggregated chromatin. The nucleoli were unique and barely perceptible. The neoplasms were interpreted as leiomyomas.

Histochemistry and immunohistochemistry (IHC)

MT staining provided clearer identification of areas with greater deposition of collagen fibers, mainly those surrounding and interspersing renal cystadenocarcinomas (Dogs 1, 4, 6-8, 10, 11), and those with mild fibrosis surrounding mainly cystadenomas (Dogs 3 and 5). Sequential sections of these cases were PR stained and observed under polarized light. It was observed that areas with fibrosis, regardless of the degree of severity, were composed of type 1 collagen. Likewise, in the areas of nodular dermatofibrosis, collagen fibers were intensely stained in blue under MT staining (Fig.3D) and type 1 collagen was identified under PR staining (Fig.3E).

IHC using the anti-pancytokeratin antibody applied on sections of the kidney showed irregular or absent immunostaining of the epithelial cells of the cysts and neoplasms. The most intensely immunostained cells were observed in areas with cystadenocarcinoma in a more solid pattern in Dog 10 (Fig.3F), as well as in metastasis in the omentum in this case.

DISCUSSION

The age of the 11 affected dogs and their mean age were very similar to those observed in another study conducted with 43 German Shepherd dogs, which is considered as the first detailed pathological description of this hereditary syndrome (Lium & Moe 1985). A male:female ratio of 2.67:1 was established for the 11 dogs of this study. Most reports suggest a predominance of males, in a ratio of 2:1 (Meuten & Meuten 2017). It is worth mentioning that of the 379 German Shepherd dogs necropsied during the study period, 54.89% were male and 45.11% were female.

Clinical recognition of RCND syndrome is not always easy. Based on clinical reports, only two cases had both the observation of urinary tract disease and cutaneous lesions compatible with the syndrome (Lium & Moe 1985). Six cases had signs indicative of urinary tract disease alone (or its consequences) and two dogs showed cutaneous nodules (perhaps renal disease had not yet manifested in these cases). Nonspecific clinical signs, confused with other diseases, may be factors that make clinical suspicion of the RCND syndrome difficult, and the diagnosis is often established only at necropsy and histopathological examination.

Eight dogs had some signs indicative of urinary tract disease (hematuria, urinary retention, azotemia and/or masses in the renal region), but others showed only secondary changes to chronic kidney disease (progressive weight loss, dyspnea, anorexia, vomiting, polydipsia or anemia). Similar clinical signs were observed in a study conducted with 51 dogs with this syndrome, where polydipsia and hematuria (macroscopic) were described in 25% of the cases. Severe clinical signs, including depression, fever and anorexia, were observed in 22% of the dogs. Two-thirds of these animals had peritonitis (sterile) caused by rupture of renal cysts. Pain and dyspnea were due to peritonitis (resulting from cyst rupture) and/or metastases (Moe & Lium 1997).

In only four cases there was description of cutaneous nodules in clinical reports, and in Dog 3, recurrent cutaneous nodules were the reason for the consultation, although they were detected at the necropsy of the 11 dogs studied. Skin lesions were the main reason for owners to take their dog to the clinic in 37% of the cases in a study conducted with 51 dogs with RCND syndrome (Moe & Lium 1997). In the present study, it can be inferred from the description of necropsy that, in some dogs, perhaps because they are small or multiple, but very small in the middle of the long coat, the nodules were not detected or clinically mentioned. Skin lesions were more frequent in the limbs, head and trunk, in descending order of frequency, in this survey. In another study (51 dogs), the most frequent sites of the lesions were limbs and head, and the authors emphasized that the nodules were often difficult to observe (Moe & Lium 1997).

Regarding renal changes at necropsy, the main characteristics were bilateral lesions, presence of multiple cysts of different sizes, and of neoplastic masses contained in cystic cavities, usually necrotic, destructive, and with hemorrhage or, occasionally, as solid masses. Neoplastic cysts and masses often increased kidney size and completely altered its shape. The same characteristics have been described in other studies on this syndrome (Lium & Moe 1985, Moe & Lium 1997) and were considered as indicative of primary multicenter origin (Lium & Moe 1985). The neoplastic lesions observed in the RCND syndrome differ from the sporadic forms of renal carcinomas, which are usually unilateral and single, with occasional metastases to the contralateral kidney (Lucke & Kelly 1976, Baskin & De Paoli 1977, Lium & Moe 1985, Meuten & Meuten 2017).

The simultaneous presence of cysts and the cystic nature of the neoplasms of this syndrome are different from other renal tumors described in dogs (Meuten & Meuten 2017). Renal cysts increase in size with age, and they also seem to present a tendency to malignant transformation of renal lesions with advanced age in dogs predisposed to this syndrome (Lium & Moe 1985). Cysts may be the initial lesion of this disease, and they seem to progress through stages of hyperplasia, adenomas, and adenocarcinomas (Meuten & Meuten 2017). In several cases, the presence of small cysts and the intermediate stages (hyperplasia and/or cystadenoma) of this pathological *continuum* until cystadenocarcinoma were observed. In only two cases the most advanced lesion was described as cystadenoma, without morphological evidence of intratumoral malignancy. Progression of the pathological *continuum* (from cysts to cystadenocarcinoma) would be influenced by genetic factors, with cists of purebred German Shepherd from certain strains prone to malignant transformation more rapidly (White et al. 1998).

According to the histological classification of mesenchymal tumors of the skin and soft tissues in domestic animals of the World Health Organization (Hendrick et al. 1998), nodular dermatofibrosis has been considered as part of a rare syndrome, described mainly in German Shepherd dogs (but may occasionally affect other breeds), where multiple fibrous nodules are observed in the dermis and subcutaneous tissue. According to this classification, these lesions are histologically differentiated from collagenous hamartomas because they are not limited to the superficial dermis and have normal or hyperplastic cutaneous adnexa within the redundant collagen. They are also differentiated from fibromas, which are benign neoplasms of mature fibrocytes producing abundant collagen, in which the fibers are arranged in interlocking fascicles or, less frequently, in swirls. All dogs in this study had predominantly multiple cutaneous nodules in the limbs, as well as in the head or trunk. This distribution has also been observed in studies conducted with larger samples on German Shepherd

dogs (Lium & Moe 1985, Moe & Lium 1997) and with dogs of other breeds (Marks et al. 1993, White et al. 1998, Meuten & Meuten 2017, Serakides & Silva 2017).

Collagenous lesion in the skin and desmoplastic fibrous reaction in renal neoplasms are noteworthy in RCND. In the present study, it was possible to observe that, in both lesions, collagen was dense and showed characteristics indicative of type I staining. This type of collagen is considered mature (Junqueira et al. 1979), and its presence in tumoral desmoplastic tumoral reactions has been studied in other types of carcinomas, including esophageal squamous cell carcinomas (SCC) in cattle (Faccin et al. 2017) and pancreatic ductal adenocarcinomas in humans (Armstrong et al. 2004, Shields et al. 2011). The role attributed to collagen in the desmoplastic reaction has been somewhat contradictory, ranging from acting as a barrier to tumor invasion to an association of its greater expression with a worse prognosis and increased metastatization in human neoplasias (Shields et al. 2011). Type I collagen has been associated with proliferation of neoplastic cells in human pancreatic cancer, and was also observed that desmoplastic reaction, through collagen, promoted a malignant phenotype of the neoplastic cells, resulting in a worse prognosis for the host (Armstrong et al. 2004). The observation of markedly collagenous desmoplastic reaction (caused by type I collagen) in renal cystadenocarcinomas contrasted with the mild fibrous reaction in the cystadenomas of this study, suggesting the need for further studies addressing the existence or not of a prognostic role of this intratumoral reaction in RCND.

Several theories for the pathogenesis of renal and cutaneous lesions in RCND syndrome have been postulated. White et al. (1998), when studying this syndrome in dogs of other breeds, summarized these theories as follows: one hypothesis is that dermatofibrosis is a paraneoplastic syndrome secondary to renal neoplasia; the other theory postulates that they are two different diseases that arise independently and are united by a common hereditary mechanism. Considering the fibrosis observed in the histological evaluation of the kidneys in the cases studied by these authors, they proposed a theory of concomitant onset of fibrosis in the skin and kidney; renal fibrosis would cause obstruction to the tubule flow, with consequent expansion and eventual formation of cysts in the renal tubules. In the cases herein studied, fibrosis seemed to be associated mainly with neoplasms, and may not have been associated with the genesis of cysts through obstructive mechanisms.

Only one of the three bitches in this survey presented uterine leiomyomas. These have also been frequently described in bitches with RCND (Lium & Moe 1985, Moe & Lium 1997); however, a pathogenetic mechanism for this concomitant neoplasm has not yet been proposed.

It is worth noting that in seven cases, almost all of the renal parenchyma was replaced by cystic and neoplastic cystic and necrotic masses; however, only two dogs showed extra-renal lesions of uremia at necropsy; a larger number of dogs with renal insufficiency and, consequently, uremic lesions would be expected. In a study conducted with 43 German Shepherd dogs with RCND, uremia was only observed in two cases (Lium & Moe 1985). The absence of extra-renal lesions of uremia in a larger number of cases is an intriguing finding, considering the marked renal morphological changes observed in the vast majority of the kidneys analyzed in this study.

The literature surveyed showed that a specific immunohistochemical panel has not yet been established for the epithelial cells that comprise the cysts and renal epithelial neoplasms of this syndrome. For renal carcinomas of sporadic occurrence (excluding RCND), it is known that double IHC staining for cytokeratin and vimentin, uromodulin, Pax8, napsin A, and neprilysin confirms the tumor originates from kidney cells (Meuten & Meuten 2017). In the present study, pancytokeratin immunostaining was not constant in all cases. An IHC study conducted with young dogs with early RCND lesions demonstrated strong staining for cytokeratin (broad spectrum) in the epithelium that lined the renal cysts in the dogs assessed, indicating that the epithelium had a distal tubular or collecting ducts origin, because the normal distal epithelial tubule cells presented intense staining for cytokeratin, whereas normal proximal tubular cells showed weak or no staining (Moe et al. 2000).

CONCLUSIONS

This study enabled identification of the prevalence and main clinical-epidemiological and morphological characteristics of renal cystadenoma/cystadenocarcinoma-nodular dermatofibrosis syndrome (RCND).

It also demonstrated that it is not always easy to clinically recognize this syndrome, but its peculiar characteristics (renal neoplasms and nodular dermatofibrosis) observed in the gross and microscopic evaluations allow safe morphological diagnosis. It was possible to detect all phases of a possible pathological *continuum* of the renal lesions.

Through histochemical techniques, it was possible to identify the presence of type I collagen in both cutaneous and renal lesions and consider its possible involvement in the pathogenesis of renal cystadenocarcinomas.

Immunohistochemistry (IHC) showed partially satisfactory results in the staining of epithelial cells of renal cysts and neoplasms for pancytokeratin.

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Dirofilaria immitis infection in dogs in Algodoal Island, Brazilian Amazon[1]

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ABSTRACT.- Moreira H.R., Madeira E.A.O, Lima Cunha D.N., Scofield A., Góes-Cavalcante G., Abel I., Guimarães R.J.P.S. & Fernandes J.I. 2019. ***Dirofilaria immitis* infection in dogs in Algodoal Island, Brazilian Amazon**. *Pesquisa Veterinária Brasileira 39(7):510-515*. Instituto de Medicina Veterinária, Universidade Federal do Pará, Rodovia BR-316 Km 61, Bairro Saudade, Castanhal, PA 68740-970, Brazil. E-mail: vetjulio@yahoo.com.br

Dirofilaria immitis, a parasite that mainly infects domestic or wild canids, but can infect felines or humans as well, is frequent in many Brazilian areas. The main objective of this research was to determine the prevalence of natural canine infection at the Algodoal-Maiandeua Island complex, in the coastal region of the state of Pará, Brazil. A total of 67 dogs were sampled for blood microfilariae detection and for *D. immitis* DNA detection. Microfilaria and *D. immitis* DNA could be detected in 35.8% (24/67) of the animals. In one dog's sample no microfilariae were detected, but the PCR was positive, suggesting that either larvae recently were eliminated or adults died shortly before sample collecting. Therefore, it can be concluded that the occurrence of *D. immitis* is a health threat for domestic and wild canids at the Island of Algodoal, as well as for feline or human health.

INDEX TERMS: Occurrence, canids, *Dirofilaria immitis*, infection, dogs, Algodoal Island, Brazilian Amazon, heartworm, PCR.

RESUMO.- [**Infecção por *Dirofilaria immitis* em cães na Ilha de Algodoal, Amazônia brasileira.**] *Dirofilaria immitis*, um parasito que infecta principalmente canídeos domésticos ou selvagens, embora também possa infectar felinos e humanos, é frequente em muitas áreas do Brasil. O objetivo deste estudo foi determinar a ocorrência da infecção natural em cães provenientes do complexo da Ilha de Algodoal-Maiandeua, região litorânea do estado do Pará, Brasil. Um total de 67 cães tiveram o sangue coletado para detecção de microfilárias de *D. immitis* e seu DNA. Microfilárias e o DNA de *D. immitis* foram detectados em 35,8% (24/67) dos animais. Na amostra de um animal, não foram observadas microfilárias, mas o seu DNA foi detectado, sugerindo que as larvas tenham sido recentemente eliminadas ou os adultos tenham morrido antes da coleta da amostra. Portanto, pode-se concluir que a ocorrência de *D. immitis* é uma ameaça à saúde de canídeos domésticos no complexo da Ilha de Algodoal-Maiandeua, bem como para felinos e seres humanos.

TERMOS DE INDEXAÇÃO: Ocorrência, cães, infecção, *Dirofilaria immitis*, verme do coração, PCR, Ilha de Algodoal, Amazônia brasileira.

INTRODUCTION

Dirofilariasis is a parasitic disease caused by filarial worm *Dirofilaria immitis* (Leidy 1856), also known as the heartworm (Bolio-Gonzalez et al. 2007), affecting mainly domestic and wild canids (Batista et al. 2008). Humans and felines, which may be accidental hosts, are more susceptible to infection in areas with high prevalence of the canine disease (Garcez et al. 2006, Mirahmadi et al. 2017), which is distributed all over the globe (Bolio-Gonzalez et al. 2007, Bowman & Atkins 2009, Hou et al. 2011, Simsek et al. 2011, Montoya-Alonso et al. 2015, Bendas et al. 2017). It has been found in every geographical region of Brazil, with prevalence rates over the last decade ranging from 2.1% in Florianópolis, in the Southern state of Santa Catarina (SC), to 62.2% in Armação de Búzios, in the Southeastern state of Rio de Janeiro (RJ) (Silva et al. 2008,

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Furtado et al. 2009, Ogawa et al. 2013, Labarthe et al. 2014, Soares et al. 2014, Ramos et al. 2015).

Methods and techniques with different analytical sensitivities are employed to diagnose infection by *D. immitis*. The most common diagnosis techniques are the identification of microfilariae on light microscopy, PCR and variations of this method for DNA detection, anti-*D. immitis* antibody detection by means of the enzyme-linked immunosorbent assay (ELISA) and the detection of antigens by immunochromatography (Traversa et al. 2010, Simsek et al. 2011, Rojas et al. 2015, Shalini et al. 2018).

Infection in a given region can be influenced by different factors, such as the diversity and quantity of mosquitoes in the area, climate changes, anthropic action and the presence of animals not receiving chemoprophylaxis (Bowman & Atkins 2009, Genchi et al. 2011, Labarthe et al. 2014). In the North region of Brazil, epidemiological studies on canine dirofilariasis are still incipient. In the state of Pará, *D. immitis* infection has been reported in dogs living in coastal areas, such as the islands of Algodoal (Castro et al. 2012, Furtado et al. 2012) and Marajó (Soares et al. 2014, Argôlo et al. 2018).

The Algodoal-Maiandeua island, part of the municipality of Maracanã, in the northeastern mesoregion of the state of Pará and part of the Northeastern Coastal Plain of Pará (João et al. 2013), has approximately 23.78km^2 in area and its environment is influenced by the geographical proximity to the Amazon River. For its regional biota and fragile ecosystem - composed of sandbanks, dunes, mangroves and lakes - the island entered the status of Environmentally Protected Area (APA - State Law of Pará No. 5621, Pará 1990). The climate is warm and humid, and its population is distributed among a complex of four villages (Algodoal, Camboinha, Mocooca and Fortalezinha). The island's economy relies strongly on tourism, with an intense flow of people and domestic animals. This calls for health regulation measures, especially with regard to infections transmitted by arthropods, including *D. immitis*. The island houses a great number of free-ranging or stray dogs who do not get proper veterinary care, which favors a high incidence of these infections (Castro et al. 2012, Furtado et al. 2012).

This work aimed to identify the frequency of natural infection with *D. immitis* in dogs in the villages composing the island of Algodoal-Maiandeua.

MATERIALS AND METHODS

Study area. Algodoal or Maiandeua island (0°34'45" to 0°37'30" S and 47°32'05" to 47°34'12" W), with 3,100.34 ha, is located in the municipality of Maracanã, microregion Salgado and mesoregion Northeast of the state of Pará. The island is composed of four villages: Vila de Algodoal, Vila de Camboinha, Vila de Fortalezinha and Vila de Mocooca. The city of Maracanã is located at 165km by road from the state capital. Algodoal is accessed by river from Marudá port (city of Marapanim), a crossing which lasts approximately 40 minutes to Algodoal village (Ideflor-bio 2018).

Sample size and sampling. The communities composing the Algodoal island complex were visited from July to September 2013. The sample size was calculated with the aid of the Statcalc tool of software EpiInfo 3.5.3 considering an estimated human population of 2,050 inhabitants (Algodoal 2013), an estimated canine population of 293 dogs, 176 households with at least one dog, expected frequency of 40%, β error of 20% and α error of 0.01, which amounts to a total of 50 households for sampling. At the first stage, the proportionate stratified sampling, which considered each community as a stratum, led to the visit of 29 households in Vila de Algodoal, five in Camboinha, ten in Fortalezinha and six in Mocooca. The second stage involved systematic sampling, where households with at least one dog were included in the sample. However, in cases where these households had more than one dog, all animals were submitted to the tests.

Georeferencing and spatial distribution map. All households included in the study have been georeferenced by a GPS MAP (60CSX, Garmin, EUA) for subsequent spatial distribution of *D. immitis* infected and non-infected animals. The maps were drawn on software ArcGis 10.3 using the Copernicus Sentinel-2 images (http://open.esa.int/) from 9 August, 2017 and the 2012 soil use and cover map by the State Secretary for the Environment (SEMA). The coordinates of residences without infected animals, as per the techniques employed in this study, were represented as green dots on the map, whereas those with at least one infected animal were represented as red dots.

Sample collection. A total of 5mL of blood was collected from the cephalic vein of each animal and stored in tubes containing ethylenediamine tetra acetic acid (EDTA), with a final concentration of approximately 1.8mg/mL of blood.

Techniques for microfilaremia detection. All samples were submitted to three techniques for detection of microfilariae: fresh and thick blood smears and the Knott technique modified by Newton & Wright (1956), which was also used to identify the microfilariae found. In sum, the fresh blood smear technique consists in using a drop of blood with anticoagulant on a microscopy slide covered by a glass cover slip of 10×10mm. The thick blood smear technique uses two drops of blood on a microscope slide, spread with the aid of microhematocrit tubes in circular movements and air dried by manual movement. The slides were examined on an optical microscope with magnification of 400× (De Carli & Tasca 2007).

The Knott technique modified by Newton & Wright (1956) is performed by diluting 1mL of blood into 9mL of formalin 2% with subsequent centrifuging (1.500rpm for 5min). Then, the supernatant was discarded and the precipitate examined between slide and cover slip by light microscopy (magnification of 100 or 400×) for detection and morphological identification of microfilariae. Distinction of the microfilariae was based on the evaluation of their morphological characteristics, especially in the anterior and posterior ends, according to Genchi et al. (2007). The samples containing immature forms of *D. immitis* were registered as such.

Polymerase chain reaction (PCR). A commercial kit (AxyPrep™ Multisource Genomic DNA Miniprep Kit -Axygen-Biosciences, EUA) was used to extract the genomic DNA of the dogs' blood samples. The procedure was done according to the manufacturer's instructions. All samples were submitted to a PCR using primers 12SF (5'-GTTCCAGAATAATCGGCTA-3') and 12SRdeg (5'-ATTGACGGATG(AG)TTTGTACC-3'), which amplify a product of 500 base pairs (bp) in the region of gene 12S rDNA, highly conserved for nematode species (Casiraghi et al. 2004). The samples tested positive in the first PCR were submitted to a second PCR using primers 12SF2B (5'-TTTTTACTTTTTTGGTAATG-3') and 12SRdeg (5'-ATTGACGGATG(AG)TTTGTACC-3'), which amplify a product of 204bp in the region of gene 12S rDNA of *D. immitis* (Gioia et al. 2010). The protocols were used according to Gioia et al. (2010) with modifications.

The amplification solution used for the reactions was made up of 2.5mM of Buffer (100mM of Tris-HCl, pH 8.5 and 500mM of KCl), 1.5mM of $MgCl_2$, 0.5mM of each dNTP (dATP, dGTP, dCTP and dTTP), 1pmol of each primer, 0.5 unit of Taq DNA polymerase

(Ludwig Biotec®) and 5µL of DNA (~43.4ηg/µL), amounting a final volume of 25µL.

The reactions were performed in a thermocycler (Veriti 96 Well Thermal Cycler - Applied Biosystems®, USA) using a temperature protocol with an initial stage of denaturation at 92°C for 1min, followed by 40 cycles of 92°C for 30s, 49°C for 45 s and 72°C for 1min, with a final extension of 72°C for 10min.

All reaction products were analyzed by 2% agarose gel electrophoresis and staining with ethidium bromide (5mg/mL). The size of amplified products was estimated using a pattern of 100 base pairs (Ladder 50 pb-Ludwig Biotec®) and a transilluminator coupled to a photodocumentation system was used for visualization (Quantum-ST4 1000/26M).

Statistical analysis. The dogs were considered infected when the microfilariae species was identified using the modified Knott technique or when the *D. immitis*-specific PCR was positive. In order to evaluate the association of the variables used in this study with infection by *D. immitis*, the data obtained was submitted to an exploratory analysis and subsequently to chi-squared and Fisher's exact test (Sampaio 2002), using the Statcalc tool of software EpiInfo 3.5.2 with a level of significance of 95% ($p \leq 0.05$).

RESULTS

Sixty-seven (67) animals from six months of age were included in the study and stratified per village as follows: 40 dogs from Vila de Algodoal, five from Camboinha, ten from Mocooca, and 12 from Fortalezinha (Fig.1), where 38 were male and 29 female. Almost all animals were mongrel (66/67) and one animal was a Pit Bull Terrier. The study included animals aged from six to 12 months old (21/67), one and three years old (32/67) and above three years old (14/67).

Of the 67 blood samples collected, 35.8% (24/67) were considered to have been taken from animals infected with *Dirofilaria immitis* (Table 1). Vila de Camboinha was the only study location where infected animals were not identified. Vila do Algodoal presented the highest rate of infected animals, 47.5% (19/40), compared to the infection rate among dogs from Fortalezinha or Mocooca, 25% (3/12) and 20% (2/10) respectively, although there was no statistical difference ($x^2=3.73$, $p=0.1549$, $GL=2$).

Male dogs were more infected (47.4%, 18/38) than females (20.7%, 6/29) ($x^2=4$, $p=0.0455$, $GL=1$). Younger dogs, regardless of their gender, were less infected ($x^2=6.34$,

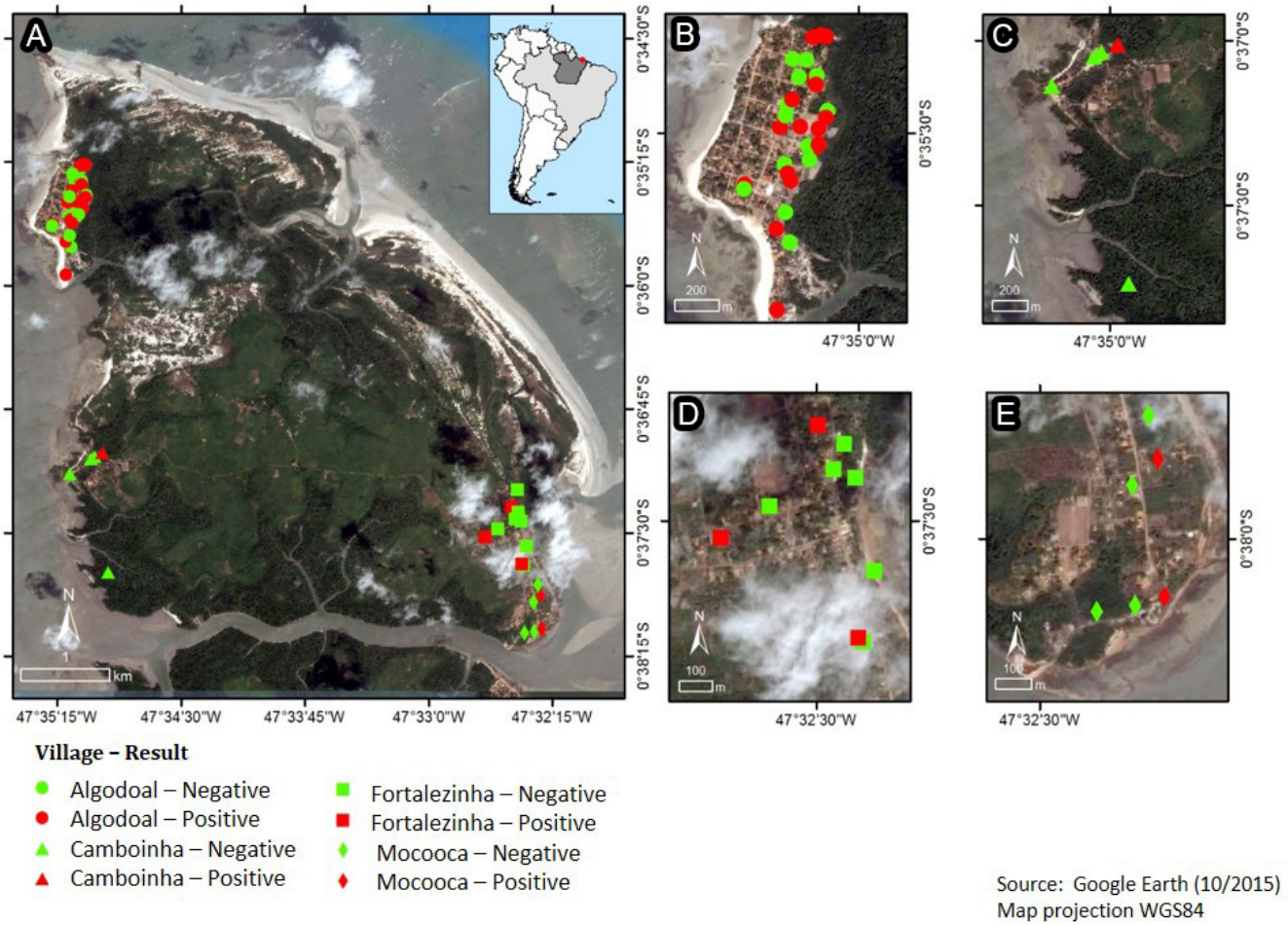


Fig.1. (**A**) Map of Algodoal island using the images of Copernicus Sentinel-2 from August 9, 2017 and data on soil use and cover provided by SEMA. Each marker represents one household included in the study, according to the area studied. Red markers represent households with at least one infected animal; green markers represent households without infected animals in the villages of (**B**) Algodoal, (**C**) Camboinha, (**D**) Fortalezinha and (**E**) Mocooca.

Table 1. Comparative results between microfilaria detection methods: modified Knott (K), thick bead (TB), fresh blood (FB), and detection of nucleic material (PCR) of *Dirofilaria immitis* microfilariae in dogs on Algodoal Island, Pará state, from July to September 2013

	Method			
	K, TB, FB and PCR	K, TB, and PCR	K and PCR	Total*
Positive	19	4	1	24
Negative	42	0	0	42
Total*	61	4	1	66

* One sample did not present microfilariae, but PCR was positive.

Table 2. Number of dogs ranked by gender and age regarding the occurrence of *Dirofilaria immitis* microfilariae on Algodoal Island, Pará state, from July to September 2013

Number of dogs	Female			Male			Total
	<1 year	>1 year and <3 years	>3 years	<1 year	>1 year and <3 years	>3 years	
Infected*	0	2	4	3	12	3	24
Free	8	10	5	10	8	2	43
Total**	8	12	9	13	20	5	67
% Infected		16.7[a]	44.4	23.1	60[b]	60	35.8

* Modified Knott technique, ** superscript different letters indicate x^2=4.1 and p=0.04295.

p=0.0420, GL=2). Although no female dog below one year old was infected among the study population, females were less infected than males only in the age range from one to three years old (x^2=4.1, p=0.0429, GL=1) (Table 2).

Of the 24 blood samples presenting microfilariae with *D. immitis* morphology, young forms with characteristics of *Acanthocheilonema reconditum* were also detected in 16 animals (66.7%). All animals with microfilariae also presented amplified DNA with primer pair 12SF and 12SRdeg and primers 12SF2B and 12SRdeg (24/24). On the other hand, in a sample without microfilariae, *D. immitis* DNA was detected by means of the same methodology. The fresh blood smear technique failed to detect microfilariae in five samples, whereas the thick blood technique failed to detect it in one sample (Table 1).

DISCUSSION

Canine infection with *Dirofilaria immitis* has been studied across the five regions of Brazil with different infection rates recorded in 14 out of 26 states (Bendas et al. 2017). Therefore, it is known that rates can vary over time and according to environmental or socioeconomic conditions (Labarthe et al. 2014). The most widely studied area in the state of Pará is the island of Algodoal, a touristic destination of the region where domestic animal health services are incipient. Vila do Algodoal is the village presenting the largest human resident population, in addition to being the gateway to the island; therefore, it concentrates the largest canine population. Most of the animals infected with *D. immitis* were found in this village (79,2%), which is likely to be due to the sample size. The prevalence rates show that infected dogs were found in all of the different villages of the island, except for the smallest one, Camboinha. The sample size was influenced by the size of each village, so that no difference was observed among prevalence rates. Nevertheless, it was observed that 35.8% of the dogs in Algodoal island have microfilaremia of the *D. immitis* species, which indicates that all dogs residing or visiting the island should receive specific chemoprophylaxis. Additionally, previous reports have shown the need to use prophylaxis upon reporting that 46.8% (Furtado et al. 2012) and 42.4% (Castro et al. 2012) of the dogs in Algodoal village had microfilaremia in January and March 2012 respectively. As this study was carried out more than one year after the first records, in which prevalence rates for Algodoal village were similar to the findings of this study (47.5%), it may be inferred that animal welfare in the island has not improved in spite of the work done.

The findings of this study and the ones published in the islands of Algodoal (Castro et al. 2012, Furtado et al. 2012) and Marajó (Furtado et al. 2009) suggest that these islands in Pará have favorable environmental conditions for the transmission of *D. immitis* infection. It may be suggested that the most prominent of these conditions are, as in other regions of Brazil (Paiva 2009): high temperatures and relative humidity maintained throughout the year, an extensive area of preserved nature and the rare characteristic of sediment deposition along the coast in areas juxtaposed to the Amazon River, forming small sand strips.

In Brazil and elsewhere, filarial co-infection in dogs with microfilaremia has been diagnosed, where the species detected most often are *Acanthocheilonema reconditum* and *D. immitis* (Brito et al. 2001, Mar et al. 2002, Reifur et al. 2004, Argôlo et al. 2018). Co-infection with *A. reconditum* and *D. immitis* was diagnosed based on the morphological identification of microfilariae; however, due to the diversity and morphological similarity between immature forms of filarial species found in the Amazon, the morphological diagnosis must be interpreted with care, as it was not associated with molecular diagnosis and phylogenetic analysis (Argôlo et al. 2018). The fact that microfilariae were not detected by the

fresh and thick blood smear technique in five animals may reflect a low concentration of microfilariae, as the analytical sensitivity threshold of these tests is of 30Mf/mL (Bowman & Mannella 2011).

It is worth highlighting that microfilariae were not found in the blood sample of a five-year-old female dog from Algodoal village, although the PCR detected the parasite's DNA. Given that finding *D. immitis* DNA in blood samples depends on the presence of microfilariae in the sample (Rossi et al. 2010), it is possible that: i) the dog did not have the disease and adult microfilariae had died, releasing DNA; ii) the microfilariae died as a result of sample manipulation; iii) the microfilariae died, freeing the dog from the disease months before the blood sample collection, as has been observed in animals treated with doxycycline (Rossi et al. 2010).

The infection was found more often among male dogs from one to three years old than among females within the same age range, which is not in line with other Brazilian studies that report not finding any differences between genders among infected animals (Almeida et al. 2001, Silva et al. 2008, Labarthe et al. 2014). It is likely that females are cared for differently in Algodoal Island, which makes them less exposed to mosquitoes than males, or that they receive some protective treatment.

The chances of transmission via blood from infecting mosquitoes to susceptible hosts increase over time so much that young animals usually present lower infection rates (Hou et al. 2011, Labarthe et al. 2014), although in hyperenzootic areas, time of residence in the region has not influenced the prevalence rate (Labarthe et al. 2014). In addition, it must be considered that the prepatent period of the infection is of at least six months, which may disguise the results of animals under one year old (American Heartworm Society 2018).

The expressive prevalence rates of canine infection observed in the state of Pará, which vary from 32.4% to 46.8% (Castro et al. 2012, Furtado et al. 2009, Furtado et al. 2012), associated with the results of this study (35.8%), show that transmission of *D. immitis* is frequent, especially in the coastal and touristic areas of the state. Therefore, considering the importance of unified health care, especially since the parasite infects domestic or wild canids and human beings, all public health agents in the region must be committed to educating the population residing and visiting the region as to the risks of infection and prevention measures.

CONCLUSION

It may be concluded that the expressive prevalence rates of *Dirofilaria immitis* in the villages composing Algodoal Island, associated to the animals' mobility, the island's environmental characteristics and the flow of inhabitants and visitors, points to the need to implement control and prophylaxis measures, which are responsibility of healthcare professionals acting in the corresponding fields of study.

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Oral lesions and retroviruses in shelter cats[1]

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ABSTRACT.- Silva A.P., Flores M., Mazaro R., Luz F., Silva M. & Fighera R.A. 2019. **Oral lesions and retroviruses in shelter cats.** *Pesquisa Veterinária Brasileira 39(7):516-522.* Hospital Veterinário Universitário, Universidade Federal de Santa Maria, Avenida Roraima 1000, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: apsvet@hotmail.com

Oral lesions are common problems in feline medicine worldwide, and may be associated with different causes, such as infectious agents. There are only a few studies reporting the chief oral diseases and the results for retrovirus tests in shelter cats in Brazil, especially in the South region. This study aimed to identify the main inflammatory oral lesions in shelter cats and verify the test results for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infections. Forty-three felines from private shelters in the central region of Rio Grande do Sul state (RS) that presented clinically evident oral lesions, regardless of age, breed, sex, and neuter status, were used in this survey. Serological tests for FIV and FeLV were performed in all cats, and data regarding the rearing system were collected. Sixteen cats (37.2%) were reared in a free system, whereas 27 (62.8%) were kept under a restrict system. Of the 43 cats with oral lesions, 29 (67.44%) presented only one type of lesion, characterized as periodontitis (n=22, 51.16%), followed by gingivitis (n=6, 13.95%), and stomatitis (n=1, 2.32%). Concomitant stomatitis and periodontitis were found in the 14 remaining cats (100%). With respect to the test results for retrovirus infections, nine (20.93%) of the 43 felines were positive for FIV alone. Co-infection with both viruses was observed in seven cats (16.28%). No cat was seropositive for FeLV valone. None of the six cats that presented gingivitis was positive for FIV and FeLV; one cat with stomatitis was positive for FIV and FeLV; of the 22 cats with periodontitis, six (27.27%) were FIV positive and two (9.09%) were FIV/FeLV positive; and of the 14 cats that presented stomatitis and periodontitis, three (21.43%) were FIV positive and four (28.57%) were FIV/FeLV positive. As for diagnosis, 28 cats (65.1%) presented solely periodontal disease (PD), one cat (2.32%) had feline chronic gingivostomatitis (FCG) alone, and 14 (32.5%) had both PD and FCG. The results obtained show that the main oral lesions found in shelter cats in the central region of RS were gingivitis, stomatitis, and periodontitis. Periodontitis, in association or not with stomatitis, was the most frequently observed oral cavity lesion in FIV- and/or FeLV-positive cats. Other factors may contribute to installation of inflammatory oral diseases in shelter cats because most cats with oral cavity lesions tested negative for retrovirus infections.

INDEX TERMS: Oral lesions, retroviruses, shelter cats, feline medicine, periodontal disease, gingivostomatitis, immunodeficiency, leukemia, clinics.

RESUMO.- [**Afecções orais e retroviroses em gatos de abrigo.**] As afecções orais são problemas comuns em medicina felina em diferentes locais do mundo e podem estar relacionadas a diferentes causas, como agentes infecciosos. Poucos estudos foram encontrados no Brasil sobre o levantamento das principais doenças orais e dos resultados de testes para retrovírus em gatos de abrigos, principalmente na região Sul. Diante disso, o objetivo deste artigo foi identificar as principais afecções orais inflamatórias em gatos de

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abrigos e verificar os resultados dos testes para o vírus da imunodeficiência felina (FIV) e o vírus da leucemia felina (FeLV). Foram incluídos 43 felinos provenientes de abrigos privados localizados na região central do Rio Grande do Sul (RS) que apresentavam lesões orais clinicamente evidentes, independente de idade, raça, gênero e estado reprodutivo. Em todos os gatos foram realizados testes sorológicos para FIV e FeLV e obtidas informações referentes ao sistema de criação. Em 16 gatos (37,2%), o sistema de criação era livre, enquanto em 27 (62,8%) era restrito. Dos 43 gatos com lesões orais, em 29 (67,44%) foi verificado somente um tipo de lesão, caracterizado como periodontite (n=22, 51,16%), seguido de gengivite (n=6, 13,95%) e estomatite (n=1, 2,32%). Lesões concomitantes de estomatite e periodontite foram encontradas nos 14 gatos (100%) restantes. Quanto aos resultados dos testes para retrovírus, nove (20,93%) dos 43 felinos testados, foram positivos somente para FIV. Em sete gatos (16,28%) foi observada coinfecção pelos dois vírus. Em nenhum gato foi observado soropositividade somente para FeLV. Dos seis gatos com gengivite, nenhum foi positivo para FIV e FeLV; um gato com estomatite foi positivo para FIV e FeLV; dos 22 gatos com periodontite, seis (27,27%) foram FIV positivos e dois (9,09%) FIV/FeLV positivos; e dos 14 com estomatite e periodontite, três (21,43%) foram FIV positivos e quatro (28,57%) FIV/FeLV positivos. Quanto ao diagnóstico, em 28 gatos (65,1%) foi observada somente doença periodontal (DP), em um (2,32%) somente gengivoestomatite crônica felina (GECF) e em 14 gatos (32,5%) DP e GECF. Diante dos resultados obtidos, pode-se concluir que as principais lesões orais encontradas em gatos de abrigos da região central do RS foram gengivite, estomatite e periodontite; a periodontite associada ou não a estomatite foi a lesão oral mais frequente nos gatos positivos para FIV e/ou FeLV. Acredita-se que outros fatores possam contribuir na instalação de doenças orais em gatos de abrigos, já que houve predomínio de gatos com resultados negativos nos testes para os retrovírus.

TERMOS DE INDEXAÇÃO: Afecções orais, retroviroses, gatos de abrigo, medicina felina, doença periodontal, gengivoestomatite, imunodeficiência, leucemia, clínica.

INTRODUCTION

Inflammatory oral disease in cats is a complex and multifactorial condition consisting primarily of local or widespread inflammation in the oral cavity (Kornya et al. 2014). The main oral diseases observed in felines are periodontal disease (PD) (Clarke & Cameron 1998, Perry & Tutt 2015), which corresponds to 13.9% of all diagnoses for this species (O'Neill et al. 2014), and feline chronic gingivostomatitis (FCG), which accounts for approximately 72% of the odontological diagnoses (Frost & Williams 1986).

PD is mainly caused by Gram-positive aerobic bacteria, although other risk factors are associated with the prevalence and severity of periodontal lesions (Logan 2006, Perry & Tutt 2015). Other causes acknowledged in the PD and FCG etiology include dental calculus deposits, an unbalanced diet -including lack of dry foods (Studer & Stapley 1973, Watson 1994, Clarke & Cameron 1998, Logan 2006), and immunological factors (Tenorio et al. 1991).

FCG is characterized by persistent, usually severe inflammation of the gingiva and oral mucosa presenting ulcerative and/or proliferative lesions, especially in the palatoglossal arch region (Lyon 2005). FCG may be considered as multifactorial, although it is likely that infectious agents are involved (Mallonee et al. 1988, Love & Zuber 1987, Knowles et al. 1989, Tenorio et al. 1991, Lyon 2005).

Upon diagnosing oral cavity lesions in cats, the possibility of underlying systemic diseases should be considered. It is believed that oral cavity lesions occur in cats infected with the feline immunodeficiency virus (FIV) or feline leukemia virus (FeLV) due to their systemic immunosuppressing effect, which hinders the animals' ability to fight the pathogens. Oral cavity lesions are among the different clinical manifestations of retrovirus infections, and are characterized by several degrees of gingivitis, stomatitis, and periodontitis (Arzi et al. 2008).

FIV and FeLV belong to the family Retroviridae. They can cause insidious infections specific of domestic felines, and are usually disseminated in cat populations (Levy et al. 2008, Hartmann 2011). FIV is a retrovirus of the genus *Lentivirus*, which is transmitted primarily in saliva through the bite wounds (Hosie et al. 2009). FeLV is a *Gammaretrovirus* that is also primarily transmitted via saliva (Lutz et al. 2009).

Prevalence of FIV and FeLV infections varies widely according to the characteristics of the populations evaluated (Levy et al. 2006). In the United States, the prevalence of both infections is approximately 2% in healthy cats and 30% in high-risk or unhealthy felines (Levy et al. 2006).

In Brazil, research on the occurrence of FIV and FeLV infections in cats from different regions (Northeast, Central-West, South, and Southeast) and facilities (veterinary hospitals and clinics, shelters, or private homes) showed prevalence figures varying from below 2.0 to 37.5% and from 8.0 to 63.0%, for FIV and FeLV, respectively (Teixeira et al. 2007, Meinerz et al. 2010, Santos et al. 2013, Almeida et al. 2016, Barros et al. 2017, Poffo et al. 2017). However, none of these studies have identified the occurrence of the chief oral lesions in the populations tested for retroviruses.

Therefore, due to the relevance of this topic and the absence of data on the main oral cavity diseases and test results for retrovirus infection in shelter cats in the South region of Brazil, this study aimed to identify the main oral lesions and verify the test results in a feline population in the central region of Rio Grande do Sul state.

MATERIALS AND METHODS

The study sample was composed of forty-three felines from three private shelters located in the central region of Rio Grande do Sul state, Brazil, presenting clinically evident oral cavity lesions regardless of age, breed, sex, and neuter status, and with no vaccination record. Inclusion criteria for oral evaluation were based on the owners' observation of changes in the animals' behavior during feeding, such as difficulty grasping, dysphagia and/or sialorrhea.

Patients were authorized to participate in the study by the owner-manager of each shelter, and all procedures were approved by the Ethics Committee on Animal Use (CEUA) of Universidade Federal de Santa Maria (UFSM) under protocol no. 4659251017/2017.

The animals' oral cavity was inspected under general dissociative anesthesia, prepared with an association of ketamine hydrochloride 10% ($8mg/kg)^{-1}$ and xylazine hydrochloride 2% ($1mg/kg^{-1}$), both administered through deep intramuscular route.

According to the gross findings, oral lesions were classified as follows: 1) gingivitis (inflammation of the gingiva alone);

2) periodontitis (inflammation of the periodontium); 3) stomatitis (inflammation of the mucosa lining the caudal oral cavity lateral to the palatoglossal arch, extending rostrally to the gingiva, the alveolar, oral, labial and sublingual mucosae and, occasionally, to the ventral and dorsal surfaces of the tongue, including lesions compatible with FCG (Gawor et al. 2006, Reiter & Soltero-Rivera 2017). Each cat was diagnosed with PD and/or gingivostomatitis (GS) according to the lesions observed (Table 1). Non-inflammatory oral lesions, such as dental fractures and absence of teeth, were not included in this study.

Blood samples (3mL) were collected from the jugular vein following tricotomy and antisepsis and stored in tubes containing EDTA and serum separation tubes for further detection of anti-FIV antibodies

Table 1. Gender, reproductive status, age, type of oral lesion, diagnosis, and test results for retrovirus infections of the 43 felines from shelters in the central region of Rio Grande do Sul state, Brazil

Cat no.	Sex/Neuterstatus	Age (years)	Type of oral cavity lesion	Diagnosis	Retrovirus	
					FIV[a]	FeLV[b]
1	IM[c]	NI[f]	Stomatitis/periodontitis	FCG[h]/PD	+[j]	+
2	IM	NI	Stomatitis/periodontitis	FCG/PD	+	+
3	IM	NI	Periodontitis	PD[i]	+	+
4	IM	NI	Periodontitis	PD	+	+
5	NF	NI	Periodontitis	PD	+	-[k]
6	NF	NI	Periodontitis	PD	+	-
7	NF	NI	Periodontitis	PD	+	-
8	IM	NI	Periodontitis	PD	+	-
9	IF	NI	Stomatitis/periodontitis	FCG/PD	+	-
10	IM	NI	Stomatitis	FCG	+	+
11	NM[d]	NI	Stomatitis/periodontitis	FCG/PD	+	+
12	NF[e]	12	Gingivitis		-	-
13	NM	05	Stomatitis/periodontitis	FCG/PD	-	-
14	NM	09	Stomatitis/periodontitis	FCG/PD	-	-
15	NF	NI	Stomatitis/periodontitis	FCG/PD	-	-
16	NM	04	Periodontitis	PD	-	-
17	NM	05	Periodontitis	PD	-	-
18	NM	07	Stomatitis/periodontitis	FCG/PD	-	-
19	NM	07	Periodontitis		-	-
20	NF	07	Stomatitis; periodontitis	FCG/PD	-	-
21	NF	07	Stomatitis/periodontitis	FCG/PD	-	-
22	NF	07	Periodontitis	PD	-	-
23	NF	10	Stomatitis/periodontitis	FCG/PD	-	-
24	NM	09	Gingivitis	PD	-	-
25	NF	07	Periodontitis	PD	-	-
26	NM	14	Periodontitis	PD	-	-
27	NF	10	Periodontitis	PD	-	-
28	NF	14	Stomatitis/periodontitis	FCG/PD	+	-
29	NF	08	Periodontitis	PD	-	-
30	NF	10	Periodontitis	PD	-	-
31	NF	07	Stomatitis/periodontitis	FCG/PD	+	-
32	NF	08	Periodontitis	PD	-	-
33	NM	05	Periodontitis	PD	-	-
34	NF	05	Periodontitis	PD	-	-
35	NF	07	Gingivitis	PD	-	-
36	NM	05	Gingivitis	PD	-	-
37	NM	06	Periodontitis	PD	-	-
38	IM	08m[g]	Gingivitis	PD	-	-
39	NF	16	Periodontitis	PD	-	-
40	NF	15	Periodontitis	PD	+	-
41	NF	15	Periodontitis	PD	+	-
42	NF	07	Gingivitis	PD	-	-
43	NF	06	Stomatitis/periodontitis	FCG/PD	+	+

[a] FIV = Feline immunodeficiency virus, [b] FeLV = feline leukemia virus, [c] IM = intact male, [d] NM = neutered male, [e] NF = neutered female, [f] NI = not informed, [g] m = months, [h] FCG = feline chronic gingivostomatitis, PD = periodontal disease, [j] (+) = positive, [k] (-) = negative.

and FeLV antigens (SNAP Combo FeLV/FIV; IDEXX Laboratories). Sensitivity and specificity test values were 100 and 98.6% for the FeLV antigen and 99.2 and 100% for the anti-FIV antibody, respectively (Burling et al. 2017). No confirmatory laboratory tests were performed.

The cats were identified in individual records containing information on age, sex, breed, neuter status, rearing system, findings of the oral examination, and test results for retrovirus infections.

As for the age variable, the cats were classified into the following groups: kittens (≤1 year), adults (>1 and ≤9 years), and elderly (>9 years) (Martins 2016). Regarding neuter status, the animals were classified as sexually intact or neutered. Unknown ages were recorded as not informed (Table 1).

With respect to the rearing system, the cats were either freely raised with access to the streets or restricted to the shelter's facilities at all times.

Information regarding gender, neuter status, age, oral cavity lesions, diagnoses, and test results for FIV and FeLV infections are shown in percentage (%).

RESULTS

Three shelters located in three different municipalities in the central region of Rio Grande do Sul state, with a total population of 92 felines, were evaluated. Of these, 43 cats with oral cavity lesions were included in the study distributed as follows: 11 (100%) animals from Shelter 1, 27 (36.49%) from Shelter 2, and five (71.42%) from Shelter 3.

Of the 43 cats that composed the study sample, the age of 31 (72.09%) animals was informed by the owners: one (3.22%) was a kitten, 21 (67.74%) were adults, and nine (29.03%) were elderly. Their age varied from eight months to 16 years (median = 9). The owners were unable to inform the age of 12 cats (27.91%) (Table 1).

As for gender, 24/43 (55.81%) were females and 19/43 (44.19%) were males. Regarding neuter status, 8/43 cats (18.60%) were sexually intact (one female and seven males) and 35/43 (81.40%) were neutered (12 males and 23 females). All 43 felines evaluated (100%) were mixed breed (MB).

Among the 43 cats with clinically evident oral cavity lesions, 29 (67.44%) presented a single type of lesion, namely, gingivitis, six (13.95%) (Fig.1);stomatitis, one (2.32%) (Fig.2);periodontitis, 22 (51.16%) (Fig.3). Dual oral infection, stomatitis and periodontitis, was observed in the 14 (32.56%) remaining cats (100%) (Table 1).

With respect to the test results for retrovirus infections, nine (20.93%) out of the 43 felines tested positive solely for FIV and none presented solely FeLV infection. Concomitant infection (co-infection) with FIV and FeLV was observed in seven cats (16.28%) (Table 2). Out of the 27 retrovirus-seronegative



Fig.1. Mild gingival hyperemia (gingivitis) in shelter cat from the central region of Rio Grande do Sul state, Brazil.



Fig.2. Bilateral ulceroproliferative lesions in shelter cat from the central region of Rio Grande do Sul state, Brazil, in the oral caudal and lateral mucosa of the palatoglossal arches indicative of caudal stomatitis (gingivostomatitis) and presence of dental calculus in the maxillary teeth (periodontitis).

Table 2. Types of oral lesion and test results for retrovirus infections of the 43 felines from shelters in the central region of Rio Grande do Sul state, Brazil

Type of oral lesion	Cat no. [a]	FIV[b] (+)[c]	FIV (-)[d]	FIV/FeLV[e] (+)	FIV/FeLV (-)
Gingivitis	06 (13.96%)	—-	06 (22.22%)	—-	06 (22.22%)
Stomatitis	01 (2.32%)	—-	—-	01 (14.28%)	—-
Periodontitis	22 (51.16%)	06 (66.66%)	14 (51.85%)	02 (28.57%)	14 (51.85%)
Stomatitis/periodontitis	14 (32.56%)	03 (33.34%)	07 (25.92%)	04 (57.14%)	07 (25.92%)
TOTAL	43 (100%)	09 (20.93%)	27 (62.79%)	07 (16.27%)	27 (62.79%)

[a] None of the cats tested positive solely for FeLV, [b] FIV = feline immunodeficiency virus, [c] (+) = positive, [d] (-) =negative, [e] FeLV = feline leukemia virus.



Fig.3. Severe inflammation of the gingiva in shelter cat from the central region of Rio Grande do Sul state, Brazil, with gingival retraction and dental calculus deposition, evident in the 4th left maxillary pre-molar, which characterizes periodontal disease (periodontitis).

cats, six (22.22%) presented gingivitis; one (3.70%), stomatitis; 14 (51.85%), periodontitis; and seven (25.92%), stomatitis/periodontitis (Table 1).

With regard to association of oral cavity lesions with FIV and/or FeLV seropositivity, of the six cats with gingivitis, none were FIV- or FeLV-positive and one animal with stomatitis was FIV and FeLV-positive. Of the 22 felines with periodontitis, six were FIV-positive and two were FIV- and FeLV-positive. Of the 14 cats with stomatitis and periodontitis, three were FIV-positive and four were FIV- and FeLV-positive (Table 2).

PD alone was observed in 28 cats (65.1%); GS alone was found in one animal (2.32%), and DP and GS was verified in 14 felines (32.5%) (Table 1).

As for the rearing system, 16 cats (59.25%) lived freely, whereas 27 (40.75%) were raised restricted to the shelters.

DISCUSSION

Inclusion criteria for oral evaluation were based on the owners' observation of changes in the animals' behavior during feeding, such as difficulty grasping, dysphagia and/or sialorrhea - altogether, these symptoms were observed in 43 cats. Although the study initially proposed to perform oral examination in all cats, this did not occur in the entire population (92 cats) because of difficulty to seize them and lack of authorization on the part of owners.

The cats included in this study were aged eight months to 16 years (median = 9); owners were unable to inform the age of 12 cats (27.91%) (Table 2). In a study conducted by Tenorio et al. (1991), which assessed chronic oral cavity infections in cats and their association with calicivirus, FIV, and FeLV, the animals' age varied from one to 18 years, and FeLV and calicivirus positivity was observed in young animals, whereas FIV was observed in adults. Kornya et al. (2014), in determining the association of test results positive for retrovirus infections with occurrence of oral cavity lesions in cats, observed that increased age was associated with higher prevalence of inflammatory oral disease in retrovirus-negative cats. Findings of the present study do not allow association between age range and test results for retrovirus infections, as the age of most of the FIV- and FeLV-positive cats was unknown. However, all FIV-positive cats were adults or elderly, and the only FeLV-positive cat whose age was known was an adult (Table 1).

FIV is mainly transmitted through biting, where the virus present in the saliva of an infected cat is inoculated into the blood stream of a healthy cat (Sellon & Hartmann 2012, Hagiwara et al. 2016). Adult sexually intact males with access to the streets constitute the main group at risk of infection (Levy et al. 2006) because of their aggressive behavior (Hosie et al. 2009, Grace 2011). In this study, 14 (87.50%) of 16 cats (37.20%) freely reared were FIV-positive. Of these, six (42.85%) were sexually intact males, one (7.14%) was a neutered male, and three (21.42%) were neutered females. Only two (7.41%) neutered females reared restricted to shelter (n=27) tested positive for FIV. The neutered females with access to the street may have become infected because of their aggressive behavior or during mating, following a bite from an infected male, prior to their spaying.

The owners' lifestyle, hygiene measures, and socioeconomic profile may have influenced the prevalence of retroviral infections. In this study, a shelter with 11 cats had five (45.45%) FIV-positive animals and six (54.55%) FIV- and FeLV-positive animals. Another shelter with five cats evaluated in this study had two (40%) FIV-positive animals and one (20%) FIV- and FeLV-positive animal. These shelters reared cats freely, with access to the street, and the owners' socioeconomic status was apparently low. In contrast, among the felines reared restricted to the shelter (n=27), only two cats were FIV-positive. Due to the socioeconomic status of their owners, they lived under more appropriate hygiene and handling conditions compared with those in the two other shelters - most of the animals were even submitted to deworming schedules.

Cats infected with FIV present different clinical symptoms, such as chronic rhinitis, lymphadenopathy, weight loss, glomerulonephritis (Hosie et al. 2009), and chronic gingivostomatitis, which affects more than 50% of FIV-positive cats (Hosie et al. 2009, Hartmann 2011). Another study conducted with 826 cats naturally infected with FIV reported that the most prevalent syndromes associated with seropositive cats were chronic gingivostomatitis, neoplasm, ocular inflammation, and anemia (Levy 2000). In the present study, of the 15 cats with FCG, two (13.33%) were FIV-positive and five (33.33%) were FIV- and FeLV-positive (co-infection).

PD is characterized by inflammation of the periodontal tissues. Initially, it affects the gingiva (gingivitis) and then other tissues, including the cementum-periodontal ligament and the alveolar bone (periodontitis) (Logan 2006, Perry & Tutt 2015). FCG is a chronic oral disease characterized by ulcerative and/or proliferative lesions beginning in the mucogingival junction (Perry & Tutt 2015). In this study, of the 43 cats with oral lesions, 28 (65.1%) presented solely PD, one (2.32%) had solely FCG, and 14 (32.5%) showed PD and FCG. It is also worth noting that of the 15 cats with gingivostomatitis, 14 (93.3%) presented concomitant periodontal disease, which reiterates the hypothesis of PD being a cause of FCG (Gioso 2003, Winer et al. 2016).

Progression of PD is determined by the microorganism's virulence combined with the host's immunological response (Nisengard et al. 2006). Individuals with compromised immunological response present more severe periodontal diseases (Wiggs & Lopprise 1997). In humans, the immunodeficiency virus, diabetes, and stress are considered risk factors for periodontitis. In cats, it is believed that oral cavity lesions occur in individuals infected with FIV and/or FeLV because of the systemic immunosuppressing effect of these viruses, which hinders resistance to pathogens (Arzi et al. 2008). In this study, of the 42 cats with PD (Table 1), 12 (28.57%) were FIV-positive and six were FIV- and FeLV-positive (14.28%), which probably contributed to the increased severity of this lesion in seropositive cats.

The impossibility of evaluating all of the 92 cats reared in the three assessed shelters, which precluded the analysis of the retrovirus tests in cats that may not have presented oral lesions, as well as the non-identification of the age of 11 FIV- and/or FeLV-positive cats, were limiting factors of this study, which hindered association of the oral cavity lesions and test results for retrovirus infections with the animals' age range. Additionally, not performing confirmatory tests and, consequently, the possibility of false positive results, also limited the scope of this study, as mentioned by Levy et al. (2006) and Kornya et al. (2014).

This research observed that the six cats with gingivitis were retrovirus-seronegative, which suggests that this type of lesion may be related to other causes (Perry & Tutt 2015, Möstl et al. 2013). Of the 22 cats (51.16%) presenting periodontitis, six were seropositive for FIV and two for FIV and FeLV. Of the 14 felines (32.56%) presenting stomatitis and periodontitis, three were seropositive for FIV and four for FIV and FeLV (Table 2). None of the cats presenting oral cavity lesions were solely FeLV-positive. Predominance of FIV-positive cats with periodontitis, associated or not with stomatitis, coincides with the findings reported by Kornya et al. (2014), who found association of FIV-positive cats with this type of lesion. Also noteworthy is the significant rate of co-infection observed in the present study, as seven (43.75%) of 16 cats with retroviral infection were both FIV- and FeLV-positive. Conversely, Kornya et al. (2014), upon determining association of oral health with FIV and FeLV seropositivity, considered this type of condition to be uncommon.

Association of chronic oral lesions with retroviruses in felines remains controversial (Knowles et al. 1989, Tenorio et al. 1991, Quimby et al. 2008, Kornya et al. 2014, Rolim et al. 2016). Although this study did not associate oral lesions with the test results for retrovirus infections, it was verified that of 43 cats with oral cavity lesions, nine were FIV-positive and seven were both FIV- and FeLV-positive. Therefore, it is believed that retrovirus seropositivity may have contributed to and increased the severity of oral cavity lesions in these 16 cats, as the 27 (62.79%) remaining felines were seronegative and presented the same types of oral cavity lesions. Thus, even if association had been found, other factors such as overcrowding, inadequate sanitary conditions, dietary imbalance, and stress (Pedersen & Wastlhuber 1991, Foley 2012, Möstl et al. 2013) were also observed in the study population and must be considered in the occurrence of oral cavity lesions in shelter cats.

CONCLUSIONS

The results obtained show that the main oral cavity lesions found in shelter cats in the central region of Rio Grande do Sul state were gingivitis, stomatitis, and periodontitis.

Periodontitis, associated or not with stomatitis, was the most frequently observed oral cavity lesion in the FIV- and/or FeLV-positive cats.

Other factors may contribute to the occurrence of inflammatory oral disease in shelter cats because most cats with oral cavity lesions tested negative for retrovirus infections.

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

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Original Article
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Causes of mortality of seabirds stranded at the Northeastern coast of Brazil[1]

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ABSTRACT.- Mariani D.B., Almeida B.J.M., Febrônio A.D.M., Vergara-Parente J.E., Souza F.A.L. & Mendonça F.S. 2019. **Causes of mortality of seabirds stranded at the Northeastern coast of Brazil.** *Pesquisa Veterinária Brasileira 39(7):523-529.* Laboratório de Diagnóstico Animal, Departamento de Morfologia e Fisiologia Animal, Universidade Federal Rural de Pernambuco, Rua Dom Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: fabio.mendonca@pq.cnpq.br

The aim of this work was to determine the main species of stranded seabirds at the Northeastern coast of Brazil in addition to the most frequent causes of stranding and mortality. The study was conducted in a monitored area for three years (2012-2014), from the coastline of south Alagoas through north coast of Bahia encompassing 254km of coast. The seabirds found alive during the monitoring were sent to rehabilitation, clinically examined and the carcasses were removed, necropsied and histopathologically analyzed. A total of 1.347 seabirds were found stranded. Of these, 378 were found alive and sent to rehabilitation. From the 969 dead seabirds 806 were unsuitable for necropsy, being only 163 submitted to necropsy and histopathological analysis. *Calonectris borealis, Puffinus gravis* and *Puffinus puffinus* were the main seabirds stranded in the studied area. Most stranding occurred from March to June with an increase during April and May for the most species of seabirds. The main clinical signs of stranded seabirds consisted of inappetence, apathy, low body score, hypothermia, flying or movement difficulty and prolonged recumbency. Natural causes followed by infectious diseases and anthropogenic environmental factors were the main causes of death of seabirds stranded on the Northeastern coast of Brazil.

INDEX TERMS: Seabirds stranded, wild avian diseases, wildlife conservation, mortality, Brazil.

RESUMO.- [**Causas de mortalidade de aves marinhas encalhadas na costa do Nordeste do Brasil.**] O objetivo deste trabalho foi determinar as principais espécies de aves marinhas encalhadas na costa do Nordeste do Brasil, bem como as mais frequentes causas de encalhe e mortalidade. O estudo foi desenvolvido em área monitorada durante três anos (2012-2014), entre o litoral sul de Alagoas e o litoral norte da Bahia, perfazendo um total de 254km de extensão da costa. As aves encontradas vivas foram encaminhadas para reabilitação e examinadas clinicamente; as carcaças foram removidas, necropsiadas e os órgãos foram analisados por meio da histopatologia. Um total de 1.347 aves foi encontrado, encalhadas. Dessas, 378 estavam vivas e foram encaminhadas para a reabilitação. Das 969 aves marinhas mortas, 806 estavam impróprias para realização de necropsia, sendo apenas 163 submetidas à necropsia e análise histopatológica. *Calonectris borealis*, *Puffinus gravis* e *Puffinus puffinus* foram as principais espécies encontradas na área estudada. A maioria dos encalhes para grande parte das aves marinhas ocorreram de março a junho, com um aumento durante abril e maio. Os principais sinais clínicos das aves marinhas consistiram em inapetência, apatia, baixo escore corporal, hipotermia, dificuldade de voo ou movimento e decúbito prolongado. Causas naturais, seguidas de doenças infecciosas e fatores antropogênicos, foram as principais causas de mortes de aves marinhas encalhadas na costa do Nordeste do Brasil.

TERMOS DE INDEXAÇÃO: Aves marinhas, encalhe, doenças de aves selvagens, conservação da vida selvagem, mortalidade, animais selvagens.

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INTRODUCTION

The extensive Brazilian coast composes a variety of environments with different resource conditions and habitats, covering areas of high environmental relevance and priority for conservation (Brasil 2010). Coastal wetlands and marine environments constitute one of the main routes in the Atlantic corridor for the migration of seabirds (Sick 1983). The seasonal displacement of many of these birds is accompanied by records of dead individuals found on beaches (Vooren & Brusque 1999).

The large interannually variations in the number of dead or debilitated seabirds found on the beaches is a common phenomenon in many parts of the world. Food resources restrictions, intra and interspecific competition between species, predation, weather adversities, climatic events, and anthropogenic environmental changes are the main causes of stranded seabirds (Bodkin & Jameson 1991, Schrag & Wiener 1995, Daszak et al. 2001, Hamer et al. 2002, Bell 2008). In Brazil, massive mortality of seabirds has been reported on the coastal areas of Bahia (Lima et al. 2004), Southeast (Olmos et al. 1995, Martuscelli at al. 1997) and Southern (Petry et al. 2004, Bugoni et al. 2007, Faria et al. 2014).

In Bahia, there is an indication of poor body condition as an explanation, in the Southeast is reported a mortality event of young seabirds, with low weight and potential relationships with climatic aspects and in the Southern there are also evidences of climatic conditions as potential factors of the stranding. Except for Bahia, in the Northeastern coast, there are no reports about individual causes or mortalities of seabirds.

The aim of this work was to determine the main species of stranded seabirds at the Northeastern coast of Brazil in addition to the most frequent causes of stranding and mortality.

MATERIALS AND METHODS

The present work was based on the availability of samples and data for the period from April 2012 to September 2014, coming from the Regional Program for Monitoring Strandings and Anomalies (PRMEA), carried out by Petrobras and executed by the "Fundação Mamíferos Aquáticos" (FMA). Measurement of environmental impacts required by federal environmental licensing conducted by IBAMA.

All the animal procedures were approved by the conservational and regulatory Brazilian agency IBAMA (SISBIO license number 21570-3).

The study was developed along 254Km of the coastline, from Piaçabuçu, Alagoas State (-10.34455 S/-36.30185 W) to Conde, Bahia State (-12.09639 S/37.68624W) (Fig.1). This area was segmented into relatively equidistant subareas, corresponding to 7 stretches of beaches and monitored daily through the first low tide of the day. The coastline location was georeferenced, and the stranded seabirds were identified (Harrison 1983, Narosky & Yzurieta 2003, Mata et al. 2006).

The seabirds found dead were recorded and photographed. Those found alive were rescued and taken to the rehabilitation unit. The age range was determined by the beak and plumage staining patterns of young and adults (Harrison 1983, Prince & Rodwell 1994, Mata et al. 2006, Onley & Scofield 2007, Bugoni & Furness 2009); detailed clinical examination consisted of weighting, physical inspection, measurement of body temperature, evaluation of ocular

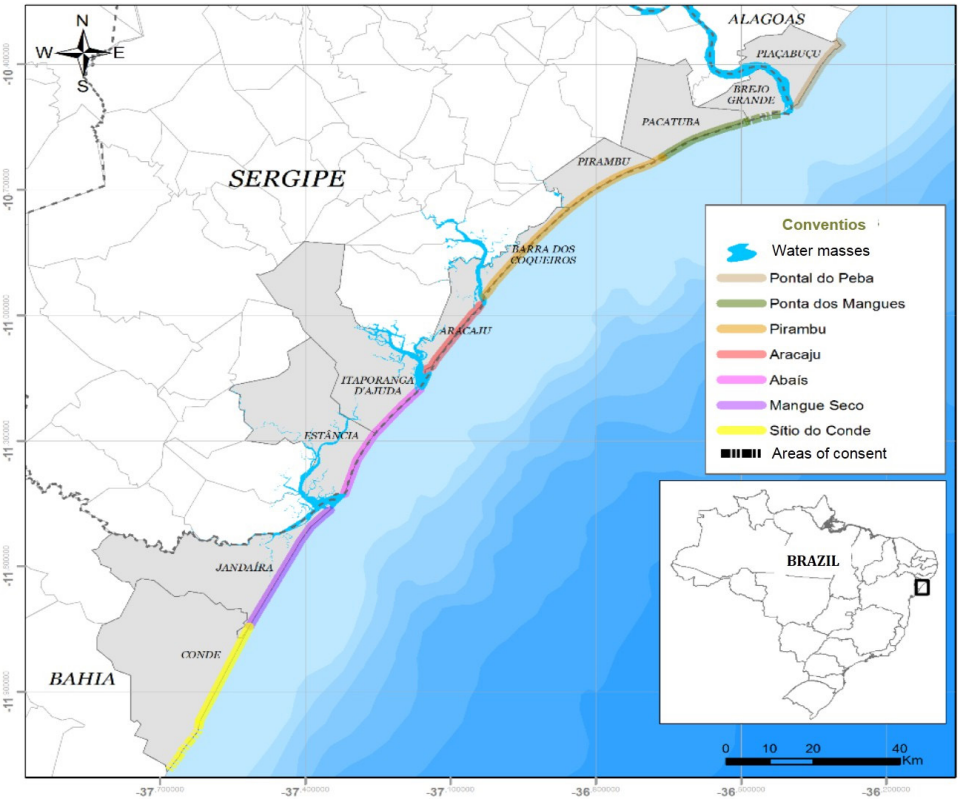


Fig.1. Brazilian northeastern coast between Piaçabuçu, Alagoas State (-10.34455 S/-36.30185 W) and Conde, Bahia State (-12.09639 S/37.68624 W).

and oral mucosa, as well as respiratory pattern, behavior and attitude. The carcasses were-classified as "very fresh", "fresh" and "moderately fresh" according to Van Franeker (2004) and examined according to Work (2000) and Munson (1999). The seabirds that died during rehabilitation were necropsied, and samples of vital organs were collected, fixed in 10% neutral buffered formalin and processed routinely for histopathology and identification of parasites in tissues samples.

RESULTS

A total of 1.347 seabirds belonging to five orders (Procellariiformes, Sphenisciformes Charadriiformes, Suliformes and Pelecaniformes) and nine families (Diomedeidae, Procellariidae, Spheniscidae, Stercorariidae, Sternidae, Scolopacidae Fragatidae, Sulidae and Ardeidae) were identified along the monitoring area (Table 1) and from these, 378 seabirds found alive were sent to rehabilitation.

Table 1. Identification, number and ages of seabirds species rescued on the coast of Northeast Brazil

Taxon	Commom name	Adult	Underadult	Juvenile	Undetermined	Total
Order Procellariiformes						
Family Diomedeidae						
Phoebetria fusca	Sooty albatross				1	1
Thalassarche chlororhynchos	Atlantic yellow-nosed albatross		1	2	5	8
Thalassarche melanophris	Black-browed albatross				2	2
Thalassarche sp.					2	2
Family Procellariidae						
Calonectris sp.					1	1
Calonectris borealis	Cory's shearwater	3	57	89	561	710
Calonectris edwardsii	Cape Verde shearwater				1	1
Pachytila sp.					1	1
Pachyptila desolata	Antarctic Prion				1	1
Procellaria aequinoctialis	White-chinned petrel	1		3	1	5
Procellaria conspicillata	Spectacled petrel	2		1	1	4
Puffinus gravis	Great shearwater	8	6	73	357	444
Puffinus griseus	Sooty shearwater		1	6	7	14
Puffinus puffinus	Manx shearwater	3	2	19	40	64
Procellariidae					2	2
Order Sphenisciformes						
Family Spheniscidae						
Spheniscus magellanicus	Magellanic penguin			8	39	47
Order Charadriiformes						
Suborder Lari						
Family Stercorariidae						
Stercorarius longicaudus	Long-tailed Jaeger	1				1
Stercorarius maccormicki	South polar skua	1			1	2
Stercorarius parasiticus	Parasitic jaeger				1	1
Stercorarius skua	Great skua			2		2
Stercorarius sp.					3	3
Family Sternidae						
Anous stolidus	Brown nody	1	1	1	4	7
Sterna hirundinacea	South American Tern				1	1
Sterna hirundo	Common tern	3	1	1		5
Suborder Scolopaci						
Family Scolopacidae						
Arenaria interpres	Ruddy turnstone	1				1
Order Suliformes						
Family Fregatidae						
Fregata magnificens	Magnificent Frigatebird				1	1
Family Sulidae						
Sula dactylatra	Maskeg booby			4	5	9
Sula leucogaster	Brown booby			1	1	2
Sula sula	Red-footed Booby			1		1
Sula sp.					3	3
Order Pelecaniformes						
Family Ardeidae						
Egretta thula	Snowy egret				1	1

Calonectris borealis (710 individuals, 52.71%), *Puffinus gravis* (444 individuals, 32.96%) and *Puffinus puffinus* (64 individuals, 4.75%) were the main seabirds stranded in this area of Northeastern coast of Brazil considering all species identified. Most seabirds (88.94%, 1.198/1.347) were of undetermined age. From the total of 969 seabirds found dead, 806 were unsuitable for necropsy due to the advanced stage of autolysis, being only 163 necropsied and sent to histopathological analysis. Of these, juveniles were 55.21% (90/163), underadult 26.99% (44/163), adults 11.04% (18/163), and 6.74% (11/163) remained undetermined. Most stranding occurred from March to June with an increase during April and June for the most species of seabirds, mainly *C. borealis*, *P. gravis* and *P. puffinus*. Stranding of *Spheniscus magellanicus* occurred only from August to September (Fig.2).

The areas with the highest rescued number of seabirds were Aracaju/Sergipe, 37.42% (504/1.347), Pirambu/Sergipe, 14.85% (200/1.347), Abaís/Sergipe, 14.62% (197/1.347) and Mangue-Seco/Bahia, 10.84% (146/1.347). The highest density of stranding occurred in Aracaju (20.16 birds/km), Abaís (5.47 birds/km), Mangue-Seco (3.95 birds/km) and Pirambu (3.77 birds/km). The average stranding across the monitored coastline was 6.09±6.27 birds/km (Fig.3).

On the seabirds clinically analyzed at rehabilitation, the main clinical signs consisted of inappetence, apathy, low body score, hypothermia, flying or moving difficulty and prolonged recumbency. Juvenile in first migration and weakened birds, followed by infectious causes, were the most important mortality events, accounting 85.8% of the cases (139/163). The seabirds generally had more than one etiological agent responsible for the weakness and consequent stranding.

Gastroenteric diseases accounted for 71.78% (117/163) of the cases. Main gross lesions were associated to the presence of parasites in the proventriculus (Fig.4A) and/or intestines in 80.34% (94/117), and plastic or others anthropogenic residues in 23.93% (28/117). From 61.70% (58/94) of samples analyzed for endoparasites, the main identified helminths were *Seuratia shipleyi*, 86.21% (50/58), *Contracaecum* sp., 12.07% (7/58), *Contracaecum pelagicum*, 3.45% (2/58) and the genus *Tetrabothrius*, 22.41% (13/58).

In most cases of moderate to severe gastric diseases there was hyperemia of the mucosa 20.51% (24/117) with hemorrhages 15.38% (18/117) and ulceration 11.96% (14/117). Moderate enteritis had similar pattern and severe enteritis were associated to mucosal congestion, 5.98% (7/117), intestinal adhesions, 2.56% (3/117), and necrosis, 2.56% (3/117). Histologically, parasitic gastroenteritis was associated to the presence of different species of helminths (Fig.4B) and heterophils infiltrations in the mucosa and submucosa, with damage to the gastric or intestines' epithelium.

Parasitic infestations were identified during inspection in 50.31% (82/163) of the seabirds, contributing to the debilitation of the birds and subsequent stranding. Of the collected samples of ectoparasites, 29.26% (24/82) were analyzed and the main species identified were *Trabeculus aviator*, 45.83% (11/24), *Halipeurus diversus*, 33.33% (8/24), *Halipeurus* sp., 12.50% (3/24) and *Naubates* sp., 8.33% (2/24).

Respiratory diseases were responsible for 26.99% (44/163) of the cases. Main clinical signs consisted of inappetence, difficult breathing, gasping and anorexia. Most diseases consisted of bacterial pneumonia and were associated to edema, congestion, opacity of air sacs and the presence of caseous material at the pleural surface. Hemorrhages 11.36% (6/44), emphysema 4.54% (2/44), pleural adhesions 4.54% (2/44) and air sacs rupture 2.27% (1/44) were present in severe cases. Histologically, 22 seabirds were analyzed.

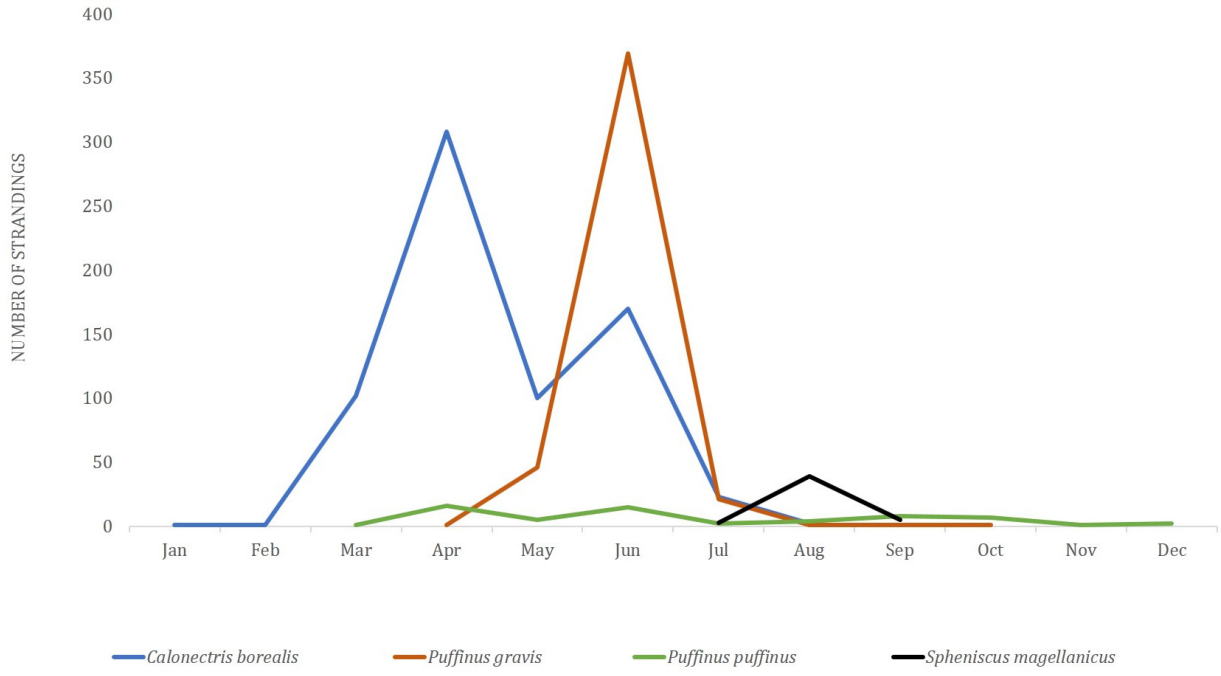


Fig.2. Seasonal distribution of stranding according to species at the studied area.

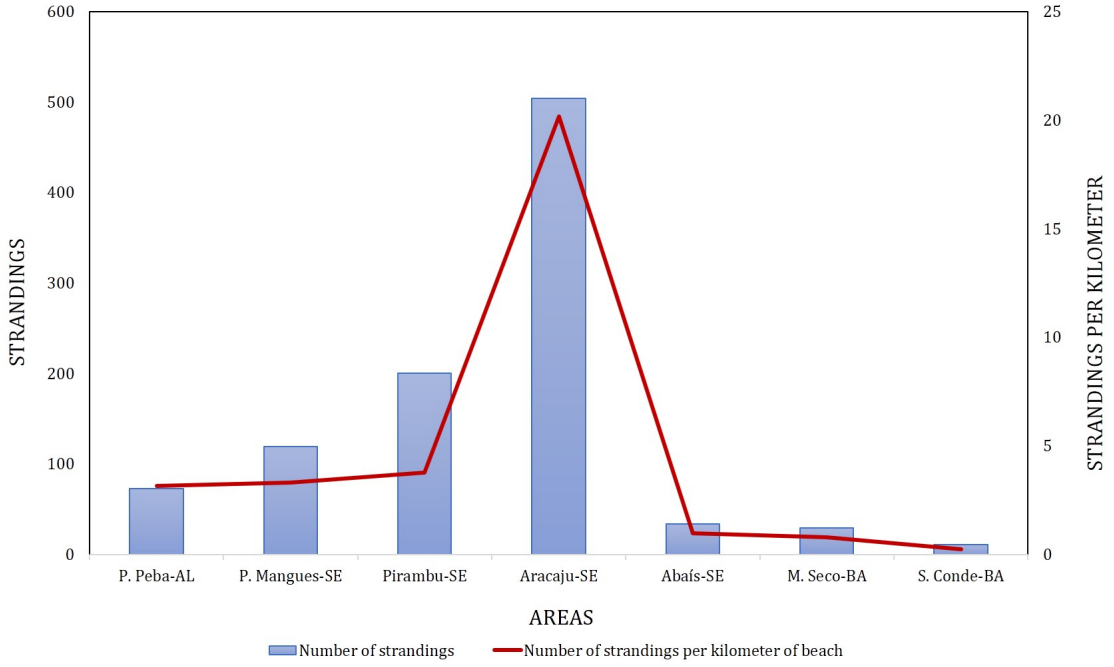


Fig.3. Numbers of seabirds per stretch of beach at the studied area.

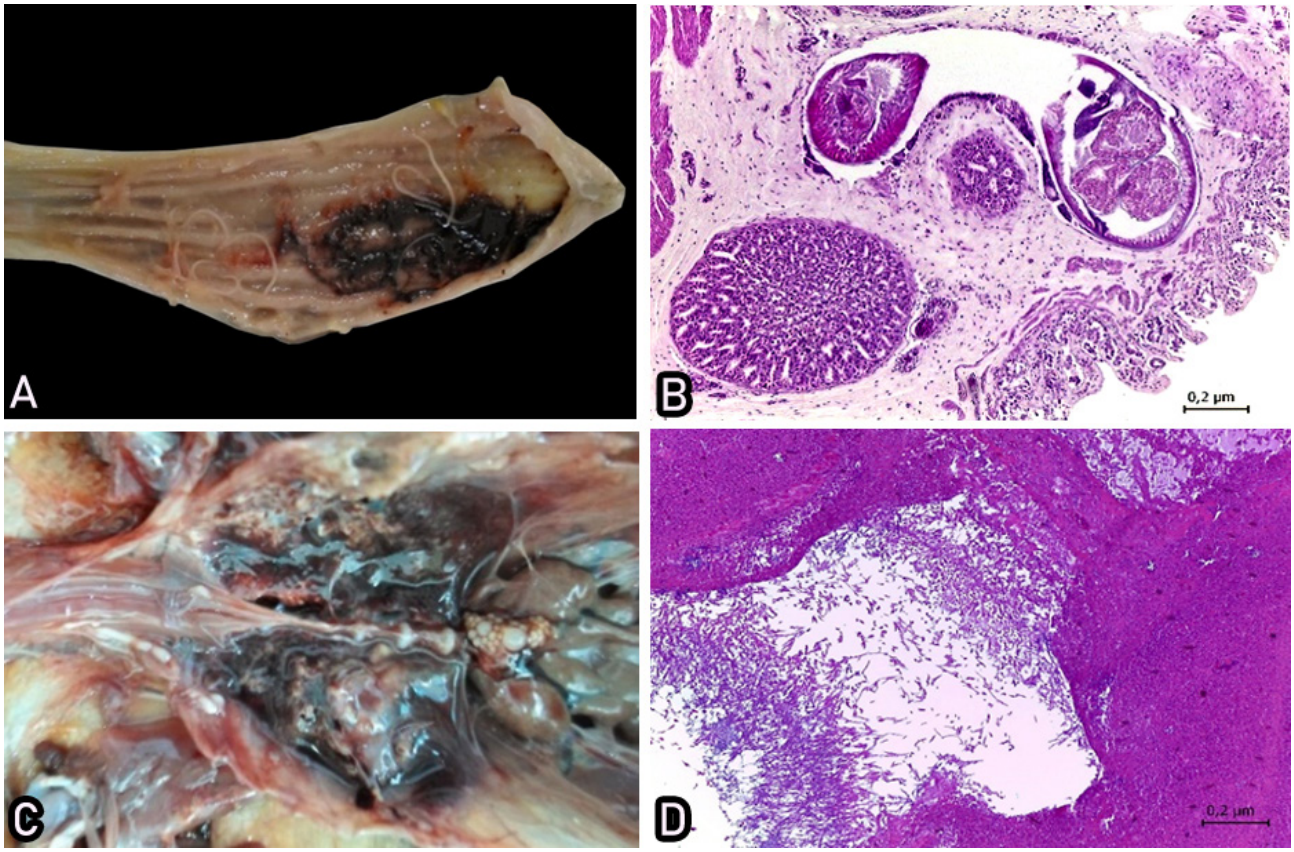


Fig.4. Macroscopic and histopathological aspects of gastric and pulmonary lesions of stranded seabirds. (**A**) Proventriculus mucosa of *Calonectris borealis* presenting areas of haemorrhage and bloody mucus, in addition to endoparasites compatible with nematodes. (**B**) Proventriculitis in *C. borealis*, characterized by discrete eosinophilic infiltrate in the submucosa with presence of cavitation containing oval structure, with external cuticle, muscular layer and internal structures such as ovary and intestine, compatible with nematode larvae. HE, obj.40x. (**C**) Granulomatous pneumonia in *Sterna hirundo* with firm and diffuse white nodules, bilateral and irregular. (**D**) Severe granulomatous pneumonia in *C. borealis* characterized by intense pulmonary consolidation and intense amount of intralesional septate hyphae. HE, obj.40x.

In acute pneumonia, there were infiltration of heterophils, monocytes, lymphocytes and the presence of cellular debris. In chronic cases, generally forming granulomatous lesions, 9.09% (2/22), there were caseous necrosis and infiltration of macrophages and giant cells.

Two cases of respiratory disease were diagnosed as fungal pneumonia. In both cases, gross lesions in the lungs were marked congestion with milliary yellow nodules (Fig.4C); air sacs were thickened with small whitish-yellow plaque-like lesions. Histologically, lungs and air sacs were replaced by disseminated granulomatous foci of caseous necrosis and cellular debris surrounded by heterophils, lymphocytes, macrophages and multinucleated giant cells with hyphal branches (Fig.4D). Also, there was pulmonary edema.

Anthropogenic interactions accounted 22.69% (37/163) of all cases and consisted mainly of ingestion of plastic/trash 54.05% (20/37). Less important cases were different kinds of traumas such as hematomas, fractures and lacerations caused by fishing tackle, 24.33% (9/37), and intoxication by oil 21.62% (8/37).

DISCUSSION

The mean of stranding along the coastline monitored was 6.09±6.27 birds/km. This data could be considered higher in comparison with other studies (Burger 2002, Harris et al. 2006). The main stranded seabird species in northeastern coast of Brazil were *Calonectris borealis*, *Puffinus gravis* and *Puffinus puffinus*. The fall (March to June) was the season with the highest number of seabirds stranding (n=1,191).

Calonectris borealis is a transatlantic migrant from the Oceanic Islands along the Iberian Peninsula (Dias 2011, Howell 2012). It occurs in Brazil throughout the year, mainly from April to August, when they return to the colonies in north Atlantic under the influence of the subtropical convergence in southern Brazil, Argentina and Uruguay (Pacheco & Maciel 1995, Sick 1997). This period also corresponds to the migration of *Puffinus gravis* to north Atlantic during the months of May to July for feeding, and to spend the non-breeding period for molting the plumage (Harrison 1983, Howell 2012).

Puffinus puffinus is a transatlantic migrant species from the northern hemisphere (Onley & Scofield 2007) which reproduces in Great Britain (Scotland, Wales), Ireland and migrates to the south Atlantic, Argentina, Uruguay and southern Brazil during the non-reproductive period (Guilford et al. 2009, Freeman et al. 2013). They stranded throughout the year, but increased between March and June and also September and November. These months corresponds to the end of the winter and spring, after the arrival of these seabirds from British islands, where they reproduce.

The predominance of stranding of these three species in northeastern coast of Brazil are also seen in southwestern and northwestern coast of the United States (Haman et al. 2013) in which most stranded birds are juveniles, probably untrained and weak to their first migration to the northern hemisphere (Vooren & Brusque 1999, Lima et al. 2004, Lee 2009).

Spheniscus magellanicus is the penguin species more frequent in Brazil, found along the coast of Rio Grande do Sul, with some wandering individuals reaching Rio de Janeiro during the winter (Sick 1997, Pinto et al. 2004). The occurrence of this species in the Brazilian Northeast is somewhat intriguing despite the historical records of carcasses found (Farias et al. 2002, Lima et al. 2004, Tavares & Nascimento 2009). In 2008, many penguins were recorded for the Northeast states, including Sergipe (Roos 2008). Other two stranded species, *Procellaria aequinoctiallis* and *Procellaria conspicillata*, deserve special attention since they are considered vulnerable.

The clinical and pathologic data indicates that environmental factors are the major cause of individual mortality of stranded seabirds in northeastern coast of Brazil. The exact causes of deaths are difficult to determine, but in most cases, it is suggested that food deprivation and severe climatic conditions leads to debilitating conditions, such as apathy, inappetence, malnutrition, dehydration, stranding, hypothermia and death (Harvell et al. 1999, 2002).

Infectious diseases were also an important cause of mortality of stranded seabirds. The number of dead seabirds was greater due to gastric or enteric parasitism and bacterial diseases. According to Brown et al. (1992) in a lot of cases, more than one etiology agent is responsible for these deaths. However, parasitic infections and bacterial pathogens in nearshore seabirds are poorly understood because these pathogens are not often documented (Muzaffar & Jones 2004), and it is unclear whether they have played a role in mortality events (Newman et al. 2007).

Several kinds of anthropogenic environmental factors were responsible for seabird's deaths and included mainly ingestion of plastic, fishing lines and hooks, fishing line entanglement and different kinds of traumas. These mortalities should be considered as an additional significant impact to seabirds, mainly due to its severities leading to death (Tasker et al. 2000, Newman et al. 2007). It is worth mentioning that no studies were conducted about the impact of light pollution on seabirds with traumas and organic pollutants on all seabirds. These results could increase the knowledge on animals affected by anthropic alterations.

CONCLUSIONS

Natural causes were the main reasons for stranding of seabirds.

The results of this study provide an important first attempt to use continuous monitoring and necropsies to understand the causes of seabird's mortality in the coast of northeastern Brazil and serves as an indicator of ocean health.

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

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Use of infrared thermography in Quarter Horse submitted to team roping[1]

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ABSTRACT.- Gerardi B., Denadai D.S., Pereira M.S., Chaves A.A., Barbosa J.P.B., Peiró J.R., Feitosa F.L.F. & Mendes L.C.N. 2019. **Use of infrared thermography in Quarter Horse submitted to team roping**. *Pesquisa Veterinária Brasileira 39(7):530-537.* Departamento de Clínica, Cirurgia e Reprodução Animal, Universidade Estadual Paulista "Julio de Mesquita Filho", Campus de Araçatuba, Rua Clóvis Pestana 793, Araçatuba, SP 16050-680, Brazil. E-mail: bi_gerardi@hotmail.com

This study aimed to verify whether the body and local temperatures change after high-intensity, short-duration exercise (team roping) and whether different pieces of training influence these changes. To this end, twelve animals, males and females, aged 3-6 years, with an average weight of 450 kg, were used. The horses were divided into two groups: regular training (RTG) and sporadic training (STG). The surface temperatures were assessed using a specific thermal camera. Temperatures of the ocular, thoracolumbar, distal tendon (thoracic and pelvic limbs) and croup regions were measured 30 min before, immediately after, and one, two, six and 24 hours after competition simulation. In the RTG, there was an increase in surface eye temperature two hours after exercise, returning to baseline level 24 hours later. In the STG, increase in eye temperature occurred immediately after exercise and returned to baseline level two hours later. Temperature of the pelvic limb tendons and croup (right side) rose immediately after exercise and did not return to baseline level 24 hours later. Team roping exercise increased the surface temperature of the distolateral thoracic and pelvic limb, croup and thoracolumbar regions in both groups and the eye temperature in the STG. Training frequency influenced the surface temperature profile in the distolateral pelvic limb, croup and thoracolumbar regions.

INDEX TERMS: Infrared thermography, Quarter Horse, team roping, exercise, horses, inflammation, thermoregulation.

RESUMO.- [Uso da termografia infravermelha em equinos da raça Quarto de Milha submetidos à prova de laço em dupla.] Os objetivos do presente estudo foram verificar se as temperaturas corpóreas e locais se alteram após exercício de alta intensidade e curta duração (prova de laço em dupla) e se treinamentos distintos podem influenciar nestas alterações. Foram utilizados 12 animais, machos e fêmeas, com idade entre 3 e 6 anos e peso médio de 450kg. Os animais foram divididos em dois grupos: treino regular (GTR) e treino esporádico (GTE). As aferições da temperatura por meio de termografia infravermelha foram feitas por uma câmera termal específica. As medições das temperaturas das regiões ocular, toracolombar, tendíneas distais (membros torácicos e pélvicos) e garupa foram realizadas 30 minutos antes, imediatamente depois, uma, duas, seis e 24 horas após a simulação de competição. No GTR houve aumento de temperatura ocular duas horas após o exercício, retornando ao basal apenas 24 horas depois. No GTE o aumento ocorreu imediatamente após o exercício e retornando ao basal duas horas depois. As temperaturas da região dos tendões dos membros pélvicos e garupa (lado direito) elevaram-se imediatamente após o exercício e não retornaram ao basal após 24 horas. O exercício de laço em dupla aumentou as temperaturas superficiais nas regiões distolateral de membros torácicos e pélvicos, garupa e região

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toracolombar de ambos os grupos e da temperatura ocular do GTE. A frequência de treinamento influenciou o perfil de temperatura superficial na região distal de membros pélvicos, garupa e toracolombar.

TERMOS DE INDEXAÇÃO: Termografia infravermelha, Quarto de Milha, prova de laço, equinos, exercício, inflamação, termorregulação.

INTRODUCTION

Brazil has a population of approximately five million equines, one million of which are intended for sports and leisure (Lima & Cintra 2016). Considering only Quarter Horse and its cross-breeds, there was a population of 460 thousand registered animals in 2017 (Associação Brasileira do Quarto de Milha 2018). The economic market associated with sport and leisure horses is of approximately USD 5.84 billion/year (Lima & Cintra 2016). Care of these equine athletes is of utmost importance, thus the monitoring of athletic performance and clinical status of these animals is essential for the development of healthy and high-performance horses.

Team roping is one of the most common modalities practiced with Quarter Horses, an event in which two mounted riders rope a steer: the first roper (header) is responsible for roping the head or horns, while the second roper (heeler) should rope the hind limbs. The competition takes eight to ten seconds on average, from the time the riders come out of the box until the steer is caught and held by stretched ropes tied to the saddles of the horses. The team that completes the task in the shortest time wins (Caiado et al. 2011). After roping the steer, the horse ridden by the header makes a 90-degree turn to the left, positioning its right thoracic limb cranially and laterally to decelerate and drag the steer, placing weight and strength on the right thoracic limb. The horse mounted by the heeler makes a quick left turn to position itself to the left side of the steer so that the hind limbs can be roped; after that, the horse stops abruptly, which can injure its pelvic limbs (Dabareiner 2011).

Infrared thermography is a noninvasive technique with potential for real-time diagnosis of local inflammation, which can lead to clinical disease (Simon et al. 2006). Through this method, it is possible to identify changes on skin surface temperature, which is directly associated with underlying circulation, tissue metabolism, and local blood flow (Eddy et al. 2001, Tunley & Henson 2004). Local temperature increase may be a result of an inflammatory process caused by increased local circulation (Simon et al. 2006) or physical exercise in response to the production of heat by muscle contractions (Hodgson 2014). Temperature measurements taken from muscle biopsy have shown positive correlations between muscle temperature and exercise duration in horses. Results showed a gradual increase in temperature with continued exercise, reaching an average temperature of 38.8°C (Lovell et al. 1987).

Thermographic image assessment of a muscle can be performed by comparing the same anatomical regions on opposite sides. Under normal situation, the images should be almost identical. Significant changes in these regions may indicate muscle injury (Yanmaz et al. 2007). In addition, this diagnostic method can detect local temperature changes even before detection by direct palpation. Significant tissue changes can be diagnosed up to two weeks prior to the occurrence of other clinical signs (Turner et al. 2001). Increased serum muscle enzyme concentration indicates muscle injury only, but not exactly which muscle or muscle group is involved. In contrast, thermography can identify the individual affected structures (Turner 2011).

Previous studies (Schaefer et al. 2007, Dunbar et al. 2009, Johnson et al. 2011) have shown that changes in temperature in the ocular region are associated with altered body temperature. Infrared thermography has been used as a quick, painless, easy-to-perform method for controlling fever in cattle with respiratory diseases (Schaefer et al. 2007). Dunbar et al. (2009) demonstrated no difference between the eye and rectal temperatures measured by thermography in deer. In horses, infrared thermography of the ocular region has proved to be a good indicator of body temperature, and when the use of maximum eye temperature revealed 74.6% sensitivity for detecting (Johnson et al. 2011).

It has been demonstrated that long-term, high-intensity physical exercises, such as cross country, enduro, and treadmill (Simon et al. 2006, Costa et al. 2009), cause changes in both body and local temperatures. Nevertheless, there are few studies reporting changes in temperature in animals submitted to team roping (Gomide et al. 2014). Therefore, the present study aimed to verify whether surface temperatures of the medial eye, distolateral thoracic and pelvic limb, croup, and thoracolumbar regions change after team roping, and whether different pieces of training can influence these changes.

MATERIALS AND METHODS

Characterization of the animals. The present study was duly assessed and approved by the Ethics Committee on Animal Use (CEUA) of Unesp, Campus Araçatuba under protocol no. 2012-01985.

Twelve clinically healthy Quarter Horses, males and females, with an average weight of 450kg, participants in the team roping modality were used in this research. The animals were submitted to similar general training programs - same intensity, velocity and duration, always conducted by the same trainer, differing only with respect to frequency. All of them also had a rest period of at least 24 hours prior to experiment commencement. The sample was divided into two groups according to training frequency: regular training group (RTG, trained five times a week), composed of six horses, three males and three females, with mean age of 3.8 years (±0.83); sporadic training group (STG, trained twice a week), comprising six horses, three males and three females, with mean age of 6 years (±1.3). Of the six animals in each group, three were mounted by headers and three were ridden by heelers. The experiment was conducted at an equine training center in the municipality of Araçatuba, Sao Paulo state, Brazil, where the animals were kept in individual stalls and fed Tifton hay and pelleted commercial feed twice daily, in addition to mineral salt once a day and water *ad libitum*.

Competition simulations were conducted on two different days, always in the morning, from 8 to 11 a.m., with mean environment temperature of 29.2°C and relative humidity of 60.2%. The simulations were performed in a covered sand track in the training center. The Quarter Horses were randomly selected; half of the animals in the RTG and STG participated in the first simulation and the remaining horses were involved in the second simulation. The animals were submitted to team roping for five consecutive times, always conducted by the same pair of riders. The simulations lasted 15min for each animal, on average. Prior to exercise, all animals underwent a warm-up period of approximately 20min: 5min at walk, 10min at trot, and 5min at gallop, in a 7m circular path. All horses were

warmed by the same trainer and were subsequently submitted to exercise without resting.

Thermography. The study was conducted during the summer, more specifically in January. Surface temperatures of the ocular, thoracic and pelvic limb tendon (superficial and deep digital flexors), thoracolumbar and croup regions were measured by infrared thermographic imaging on both sides. These temperatures were measured using a thermal camera (FLIR® i60, USA) with 0.95 adjusted emissivity, ±2% accuracy, and 0.1°C sensitivity, in real time, at a 1m distance. Temperature measuring times were as follows: prior to exercise (M0); immediately after exercise (M1); one (M2), two (M3), six (M4), and 24 (M5) hours after exercise. The animals were kept in the shade, at room temperature, both at the time of team roping simulation and during the intervals between temperature measurements. The recorded images were analyzed using specific software (FLIR® Tools, 5.5, USA), and the surface temperatures were also corrected in relation to ambient temperature and relative humidity. The maximum ocular temperatures were measured in the medial corner of the eyes. As for the region of the distal tendons of the thoracic and pelvic limbs, analyses were performed in a rectangular area that extended from the carpometacarpal to the metacarpophalangeal joints in the thoracic limb and from the tarsometatarsal to the metatarsophalangeal joints in the pelvic limb. Measurement in the croup region (upper and lower gluteal muscles) was performed on the right and left sides in a circular area, and analysis of the spine was conducted by linearity. Environment temperature and relative humidity (Table 1) were monitored before each re-calibration of the surface temperatures using specific equipment (KLIMMALOGG PRO Thermo-Hygro-Station TFA®, Germany).

Statistical analysis. Data were tested for normality and homogeneity of variances - prerequisites for analysis of variance. The data obtained for maximum eye temperature and mean temperatures of the spine, croup, and thoracic and pelvic limbs were subjected to analysis of variance (ANOVA) with repeated measurements for comparison between groups and measuring times, followed by multiple comparisons of the means by the Tukey's test at 5% significance level. Differences were considered statistically significant when $p<0.05$ and the data were processed using the SAS software (SAS Institute Inc. 2014).

Table 1. Absolute and mean values and standard deviation of environment temperature (°C) and relative humidity (%) at the different surface temperature measuring times using infrared thermography in Quarter Horses submitted to team roping: regular training (RTG) and sporadic training (STG) groups

	Animal	Environment	Measuring times					
			M0[a]	M1[b]	M2[c]	M3[d]	M4[e]	M5[f]
RTG	1	T	28.6	28.8	31.3	33.2	39	31
		RH	60	62	53	48	30	56
	2	T	26.6	28.6	31.3	33	39	31.5
		RH	70	61	53	49	49	54
	3	T	26.8	30.4	32.3	34.4	38.7	31.8
		RH	68	58	52	44	33	52
	4	T	22.7	26.4	28.1	30.5	35.4	27
		RH	80	71	63	56	45	67
	5	T	22.6	26.9	28.6	31.3	33.9	27.6
		RH	81	68	62	52	47	64
	6	T	22.5	24.5	27.1	28.8	31.8	25.2
		RH	82	76	68	61	55	70
Mean temperature		T	24.9 ± 2.7	27.6 ± 2.1	29.8 ± 2.1	31.8 ± 2.1	36.3 ± 3.1	29.0 ± 2.8
Mean relative humidity		RH	73.5 ± 8.9	66.0 ± 6.8	58.5 ± 6.7	51.7 ± 6.1	43.2 ± 9.7	60.5 ± 7.5
STG	7	T	26.7	29.6	31.7	33.6	39.2	31.6
		RH	68	62	54	45	29	54
	8	T	26.9	30.9	31.7	33.6	39.6	31.6
		RH	68	56	53	46	31	54
	9	T	27.2	30.4	32.4	34.4	38.8	31.7
		RH	66	58	52	44	31	52
	10	T	22.7	26.8	28.8	31.4	33.5	28.3
		RH	82	70	60	52	47	63
	11	T	22.8	24.5	27.4	28.2	33.4	25.5
		RH	82	76	66	59	51	70
	12	T	23.7	25.9	28.2	28.7	33.5	23.8
		RH	76	69	62	64	51	77
Mean temperature média		T	25.0 ± 2.2	28.0 ± 2.6	30.0 ± 2.1	31.7 ± 2.7	36.3 ± 3.2	28.8 ± 3.5
Mean relative humidity		RH	76.7 ± 7.3	65.1 ± 7.8	57.8 ± 5.7	51.7 ± 8.3	40.0 ± 10.7	61.7 ± 10.2

T = temperature, RH = relative humidity; [a] M0 = prior to exercise (7 to 8 a.m.), [b] M1 = immediately after exercise (8 to 9 a.m.), [c] M2 = one hour after exercise (9 to 10 a.m.), [d] M3 = two hours after exercise (10 to 11 a.m.), [e] M4 = six hours after exercise (2 to 3 p.m.), [f] M5 = 24 hours after exercise (7 to 8 a.m.).

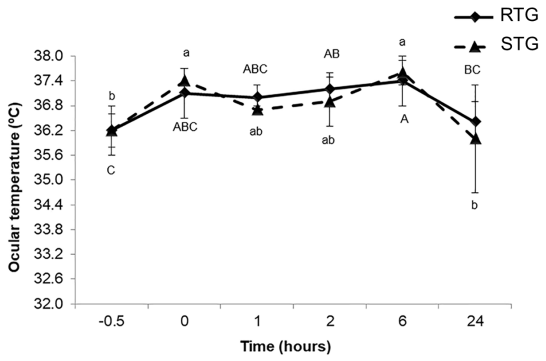


Fig.1. Maximum values and standard deviation of surface eye temperature (°C) in Quarter Horses submitted to team roping: regular training (RTG) and sporadic training (STG) groups. Means followed by the same uppercase letters do not differ over time in the RTG by the Tukey's test (p>0.05). Means followed by the same lowercase letters do not differ over time in the STG by the Tukey's test (p>0.05).

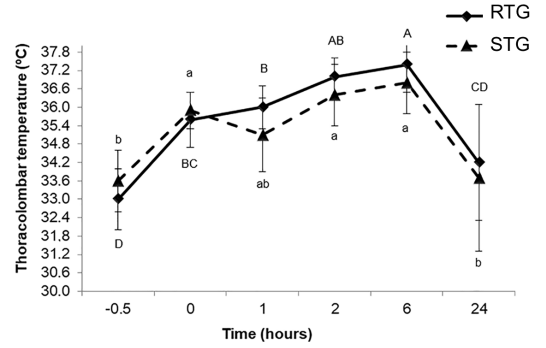


Fig.2. Mean values and standard deviation of surface thoracolumbar temperature (°C) in Quarter Horses submitted to team roping: regular training (RTG) and sporadic training (STG) groups. Means followed by the same uppercase letters do not differ over time in the RTG by the Tukey's test (p>0.05). Means followed by the same lowercase letters do not differ over time in the STG by the Tukey's test (p>0.05).

RESULTS

With respect to the ocular region, a significant increase in temperature was observed in the RTG two hours after exercise compared with 30 minutes before exercise, remaining high for further four hours. In the STG, the increase occurred immediately after exercise, returned to baseline one hour later, and remained at this level for another hour, rising again after six hours compared with that at baseline level. After 24 hours, the temperature returned to baseline in both groups (Fig.1).

Regarding the thoracolumbar region, there was a significant increase in temperature in both groups immediately after exercise. In the RTG, the temperature returned to baseline level only 24 hours after exercise, whereas in the STG, the temperature returned baseline level one hour after exercise. However, the temperature rose and remained above the baseline level between two and six hours after exercise, equaling it after 24 hours (Fig.2).

As for the croup region, the temperature profile was the same in the RTG (left side) and STG (right side). A significant increase was verified immediately after exercise, returning to the baseline level only after 24 hours. On the left side in the STG, a significant increase in temperature was observed immediately after exercise, but it returned to baseline level one hour later, rising again at two and six hours after exercise, and returning to baseline only 24 hours later. On the right side in the RTG, the temperature rose immediately after exercise and remained high for up to 24 hours (Fig.3A,B).

Concerning the tendon region of the thoracic limbs, the temperature rose significantly shortly after exercise and returned to baseline level 24 hours later on both sides, in both groups (Fig.4A,B). The same temperature changes were found in the region of the pelvic limbs in the STG, whereas in the RTG, there was no return to the baseline level in the right pelvic limb (RPL) and left pelvic limb (LPL) tendon regions 24 hours after exercise. The temperature rose significantly immediately after exercise and remained high (Fig.4C,D).

No statistically significant difference was observed between the study groups regarding the surface temperatures of the measured regions.

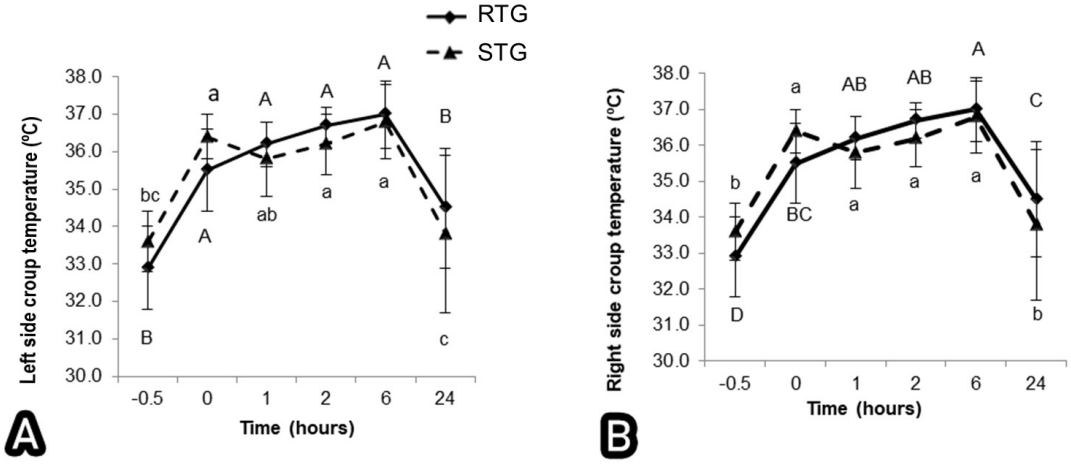


Fig.3. Mean values and standard deviation of surface croup temperatures (°C), (**A**) left and (**B**) right sides, in Quarter Horses submitted to team roping: regular training (RTG) and sporadic training (STG) groups. Means followed by the same uppercase letters do not differ over time in the RTG by the Tukey's test (p>0.05). Means followed by the same lowercase letters do not differ over time in the STG by the Tukey's test (p>0.05).

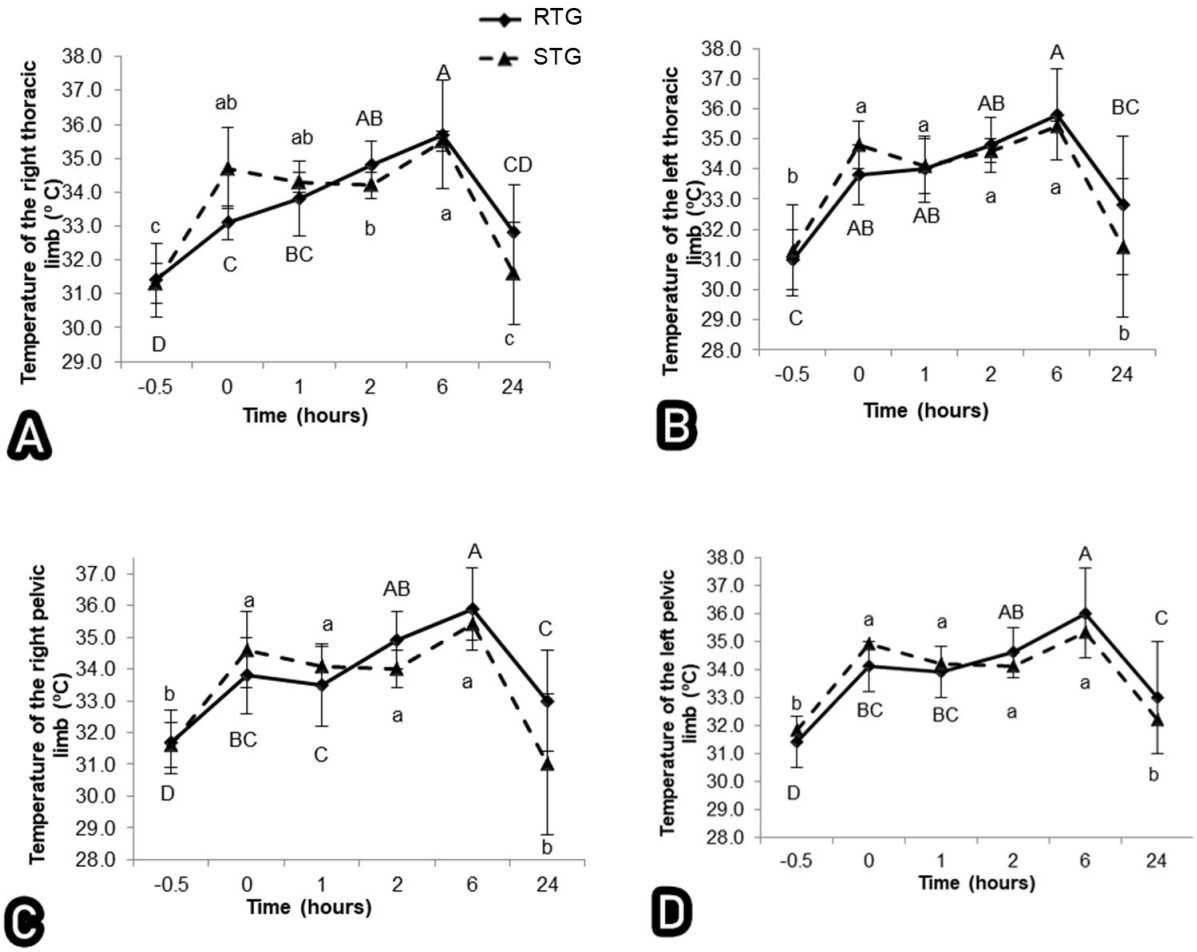


Fig.4. Mean values and standard deviation of surface temperatures (°C) in the regions of the (**A**) right thoracic limb (RTL), (**B**) left thoracic limb (LTL), (**C**) right pelvic limb (RPL) and (**D**) left pelvic limb (LPL), in Quarter Horses submitted to team roping: regular training (RTG) and sporadic training (STG) groups. Means followed by the same uppercase letters do not differ over time in the RTG by the Tukey's test (p>0.05). Means followed by the same lowercase letters do not differ over time in the STG by the Tukey's test (p>0.05).

DISCUSSION

Findings of the present study showed an increase in temperature in the ocular region after team roping simulation in the STG, suggesting that even short duration exercise can cause alteration in eye temperature. Because no significant changes in eye temperature were observed between the groups, we conclude that the training frequency did not influence the rate of central heat production. Although eye temperature is considered an alternative tool for non-invasive central temperature measurement (Schaefer et al. 2007, Dunbar et al. 2009, Johnson et al. 2011), in the present study, some measurements showed values below normal for the species; these results were probably influenced by the measurement distance. It has previously been demonstrated that measurement distances <1m present higher accuracy between rectal and maximum eye temperatures in horses (Johnson et al. 2011).

Absence of significant increase in eye temperature immediately after exercise in the RTG may be associated with a higher adaptive capacity of these animals. Animals in the RTG are believed to have a higher cardiac output than those in the STG, enabling greater redistribution of blood to organs and tissues participating in thermolysis, including the skin, respiratory muscles, and nasal mucosa (McConaghy et al. 1996), consequently promoting faster heat dissipation and not causing significant temperature change.

Decrease in temperature observed in the animals of the STG one hour after exercise was associated with the thermoregulatory activation mechanism in an attempt of these horses to avoid post-exercise hyperthermia. It is common knowledge that the capacity to modify the irrigation of different organs, adjusting it to different needs, is a characteristic of the cardiovascular system that efficiently assists with thermoregulation (McConaghy et al. 1996). The results obtained in this study show that the thermoregulatory mechanism was also efficient in the STG, since the temperatures in the thoracolumbar, croup and ocular regions returned to the baseline level one hour after the exercise. Although temporarily away from competitions and resuming training, the animals in the STG are conditioned to exercise, which explains the efficiency of their thermoregulation mechanism. According to Hodgson (2014), the faster the animal returns to baseline frequencies and temperatures, the better its athletic conditioning.

Increase in surface temperature in the ocular, thoracolumbar and croup regions two and six hours after exercise coincides

with the elevation of ambient temperature, which hinders heat exchange. Considering that one of the forms of heat exchange occurs by convection, through the movement of air on the surface of a body as during exercise, and that this heat exchange is efficient only when the temperature of the air that surrounds the animal is lower than its skin temperature (McConaghy 1994), when keeping the animals of the present study standing still, even in a shaded place, heat exchange became dependent on the movement of circulating air, which did not occur during the experiment. Therefore, absence of air circulation along with increased environment temperature at these times has hindered heat loss by convection. In addition, the increase in ambient temperature, which at certain times was higher than the body temperature of healthy horses (37.5-38.5°C), promoted heat gain in the animals of both groups. According to Robertshaw (2006), animals gain or lose heat by conduction or convection through direct contact with hot or cold substances, including air. Another form of heat gain is through radiation; even if not directly exposed to sunlight, the body surface of an animal absorbs heat from the environment around it through long-wave radiation.

According to Tanda (2016), increased ambient temperature results in increased central temperature, with consequent activation of the thermoregulatory mechanism. Although vasodilatation facilitates heat exchange of the animal with the environment through sensitive processes, the effectiveness thereof depends on the thermal gradient between the animal's body and the ambient temperature (Mccutcheon & Geor 2008).

Regarding the temperatures in the assessed regions, the results obtained evidenced heat production in all of these regions after exercise and, consequently, a rise in the local temperatures. Temporary increase in temperature occurs primarily due to the increase in muscle metabolism, as well as to increased blood flow in the region, with the aim of increasing oxygen supply and heat dissipation. In humans, for instance, blood flow may present a 20-fold increase at exercise peak compared with the resting state (Lash 1996). However, when exercise ceases, the flow tends to normalize with return of the temperature to the baseline level (Hodgson & Rose 1994). In this study, the temperatures of all analyzed areas returned to baseline level, except for the regions of the right and left pelvic limb tendons and the right side of the croup of horses in the RTG.

In the RTG, the temperature of the tendon region in the left and right pelvic limbs did not return to baseline level after 24 hours. These results are in disagreement with those reported in previous studies, which showed that temperature in the tendon region of the pelvic limbs of horses submitted to exercise on treadmill - consisting of five minutes at walk, slow trot, trot, and slow gallop, then back to 3-min trot, slow trot, and walk - returned to baseline level 45 minutes after exercise (Simon et al. 2006). In the present study, it was observed that, before 24 hours after exercise, the surface temperatures of several regions returned to the baseline level; therefore, the thermoregulation mechanism of these animals was functional. Thus, it is possible to conclude that the temperature increase in these regions is not associated with failure in the heat loss process, but probably with the greater burden on these areas demanded by team roping.

The difference between the results of the present study and those presented by Simon et al. (2006), probably lies on the greater tension on the tendon and ligament structures of the pelvic limbs in horses undergoing team roping. Animals in this sport modality are submitted to a greater burden on the pelvic tendons at the start and roping completion times. At the latter time, the horse mounted by the heeler stops abruptly after the hind limbs of the steer are roped, and the horse ridden by the header turns 180° to stop in front of the steer (Black & Dabareiner 2011). Thus, there is a considerable difference between the movements performed on a treadmill, in which animals travel in a straight line without explosive movements, and those performed during team roping.

With respect to temperature in the croup region, previous studies have shown that muscle regions, under normal conditions, have the capacity to return to baseline temperature after exercise much faster than surfaces without muscles because of their larger surface area. However, contrary to the findings by Costa et al. (2009), the temperature in the croup region (right side) in the RTG did not return to baseline level after 24 hours. During team roping, muscle contraction on the right side of the croup is more intense than that on the left side because it assists with the turning movement of the animal to the left. The high temperature in these regions is probably linked to the greater burden to which they are submitted. According to Turner (2011), myopathies of the croup are caused by tension in the long muscle, origin of the gluteus medius (in the sacroiliac region), gluteus medius body, and gluteus insertion in the major trochanter and third trochanter of the femur.

Overburden also affects the thoracic limbs of horses, since the temperatures in these regions only returned to baseline level between six and 24 hours after exercise. At the time the header ropes the steer, his horse makes a left turn and its right thoracic limbs support much of its weight, added to the strength to drag the steer. In turn, the heeler horse supports its weight on the left thoracic limb during the turn movement (Dabareiner 2011).

In the present study, temperature measurements taken 24 hours after exercise could confirm whether these specific regions need more time to return to baseline level. If this does not occur, another explanation for the presence of high temperature in these regions 24 hours after exercise may be related to localized subclinical inflammation. Comparison between the mean temperature values in the three regions and their corresponding baseline levels showed differences greater than 1°C, namely, 1.6°C for the LPL, 1.3°C for the RPL, and 1.6°C for the croup (right side). Difference of 1°C or 1.25°C between two anatomically symmetric regions is indicative of subclinical inflammation (Head & Dyson 2001, Soroko et al. 2013). According to Dabareiner et al. (2005), injuries in roping horses are associated with their function in this sport modality. Horses mounted by headers present higher percentage of distal injuries in the forelimbs, whereas in those ridden by heelers the number of injuries is larger in their hind limbs.

Thus, the maintenance of temperature in the tendon (pelvic limb) and croup regions above the baseline level may be mainly associated with the need for longer heat dissipation time in these regions, since they are the most requested during movement, and secondly with maintenance of temperature above baseline level after 24 hours, which is suggestive of localized subclinical

inflammation, early diagnosed by infrared thermography. Early diagnosis of inflammatory lesions has been reported in previous studies, and the change in superficial temperatures indicative of inflammation was verified two weeks before the animals presented with clinical signs (Vaden et al. 1980, Turner 2011). It is known that excessive and repetitive exercise can cause different types and levels of subclinical lesions in the collagenous matrix that cannot be repaired by tenocytes for various reasons, including insufficient rest period between episodes and simple overburden of cell capacity caused by high frequency or non-uniformity (Kane & Firth 2014). Therefore, the probable subclinical inflammation in these animals may be associated with repetitive effort of these structures, since these animals are trained constantly and, consequently, lack the time interval necessary for recovery.

Findings of the present study clearly show that frequent training might lead to damage to anatomical structures essential for athlete horses, such as tendons and muscle groups; therefore, infrared thermography could be used as an important tool to avoid more serious injuries. According to Turner (2011), lesions with significant clinical potential can be identified and, accordingly, the training protocol can be readjusted to prevent further injury.

CONCLUSIONS

Team roping simulation increased the surface temperature of the distolateral thoracic and pelvic limb, croup, and thoracolumbar regions in both study groups, except for the eye temperature in the RTG.

Training frequency influenced the surface temperature profile, especially in the distolateral pelvic limb, croup, and thoracolumbar regions, because of the greater burden these regions are subjected during this type of exercise.

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Original Article
Animal Morphophysiology
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Morphology and biometry of the reproductive organs of adult males of *Trachemys scripta elegans* reared in São Paulo state, Brazil[1]

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ABSTRACT.- Gradela A., Pires I.C., Faria M.D., Matos M.H.T., Costa M.M., Souza R.K.C., Milanelo L. & Franzo V.S. 2019. **Morphology and biometry of the reproductive organs of adult males of *Trachemys scripta elegans* reared in São Paulo state, Brazil.** *Pesquisa Veterinária Brasileira 39(7):538-548.* Laboratório de Anatomia dos Animais Domésticos e Silvestres, Colegiado de Medicina Veterinária, Universidade Federal do Vale do São Francisco, Rodovia 407 Km 12, Lote 543, Projeto Nilo Coelho C1, Petrolina, PE 56300-000, Brazil. E-mail: agradela@hotmail.com

Trachemys scripta elegans is an American underwater chelonian illegally marketed in Brazilian pet shops. When abandoned in nature, it compromises native species, threatening local biodiversity. However, little is known about the body development and structure of its reproductive tract. The objective of the present study was to investigate the morphology and biometry of testis, epididymis and penis, as well as the biometry of the body and secondary sexual characters in this species. Twenty-seven adult males were used aiming to contribute to preservation actions in captivity, population control, and scientific research, as well as to interspecific comparisons. Sex identification by the third claw length was effective, and the specimens presented harmonious and positive body development between mass, carapace, plastron, and height, with unimodal tendency and higher frequency of maximum carapace length at 15cm. The testes and epididymides presented biometric similarity between the antimeres and anatomical and histological structure similar to that of other species of chelonians and mammals, except for the type of epithelium. The findings suggest that there is conserved morphology between slider turtles and homology in relation to mammals. Histological similarity to the reproductive organs of other amniotes, including humans, may give rise to scientific and comparative studies, essential for the establishment of conservation strategies in reptiles.

INDEX TERMS: Morphology, biometry, reproductive organs, *Trachemys scripta elegans,* Brazil, testis, penis, epididymis, turtle, reptiles.

RESUMO.- [**Morfologia e biometria dos órgãos reprodutivos de machos adultos de *Trachemys scripta elegans* criados em São Paulo, Brasil.**] *Trachemys scripta elegans* é um quelônio subaquático americano ilegalmente comercializado em *pet shops* brasileiros. Ao ser abandonado na natureza, compromete as espécies nativas, ameaçando à biodiversidade local. No entanto, pouco se conhece sobre o desenvolvimento corporal e a estrutura do seu aparelho reprodutor. O objetivo do presente trabalho foi investigar a morfologia e a biometria dos testículos, epidídimos e pênis, a biometria corporal e dos caracteres sexuais secundários. Foram utilizados 27 machos adultos desta espécie, visando contribuir com ações de preservação em cativeiro, controle populacional e pesquisas científicas, além de comparações interespecíficas. A identificação sexual pelo comprimento da terceira garra foi efetiva e os espécimes apresentaram desenvolvimento corporal harmônico e positivo entre massa, carapaça, plastrão e altura, com tendência unimodal e maior frequência de comprimento

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máximo de carapaça em 15,0cm. Testículos e epidídimos apresentaram semelhança biométrica entre os antímeros e estrutura anatômica e histológica semelhantes à de outras espécies de quelônios e mamíferos, excetuando-se pelo tipo de epitélio. Os achados sugerem haver morfologia conservada entre os cágados e homologia em relação aos mamíferos. A semelhança histológica com os órgãos reprodutivos de outros amniotas, incluindo os humanos, pode dar ensejo a estudos científicos e comparativos, essenciais para estabelecimento de estratégias de conservação em répteis.

TERMOS DE INDEXAÇÃO: Morfologia, biometria, órgãos reprodutivos, *Trachemys scripta elegans,* Brasil, testículos, pênis, epidídimos, tartaruga, répteis.

INTRODUCTION

Introduction of invasive species is a major threat to several chelonians worldwide (Buhlmann et al. 2002) and a serious danger to maintenance of biological diversity (Primack & Rodrigues 2001). This has been observed with *Trachemys scripta elegans* (Wied 1839), also known as American water tiger or red-eared slider, a species native to North America geographically distributed from Eastern United States to Northeastern Mexico (Ernst & Barbour 1989 apud Vieira & Costa 2006). Illegal and indiscriminate trade has made this species the most commercialized in Brazil; however, in Cerrado areas, it is an exotic and invasive species that, when released in ponds, lakes, rivers and other bodies of water, threatens local biodiversity and endangers native species through competition or genetic extinction resulting from hybridization (Fonseca 2001).

Interest in studies addressing reproduction of reptiles has increased because of conservation needs. The following genera naturally occur in Brazil: *Trachemys dorbignyi* and *Trachemys adiutrix, Podocnemis unifilis* and *Podocnemis erythrocephala, Phrynops hilari, Phrynops williamsi* and *Phrynops geoffroanus, Kinosternon scorpioides.* Environmental factors and selection pressure have caused local adaptations and specific conditions in natural populations (Schluter 2000) that led to morphological and genetic variations within and between species and subspecies of the genus *Trachemys* (Daza & Paez 2007, Batistella 2008, Readel et al. 2008). In this genus, differences in body size and shape resulting from different habitats are observed among monitored populations (Pérez et al. 2010). For instance, larger females have been found in areas where hunting is less intense (Close & Seigel 1997, Daza & Paez 2007) and where there is greater rainfall (Close & Seigel 1997).

Lack of information on reptiles hinders population control, because their morphology, anatomy and physiology are unique. Therefore, knowledge about the anatomy and physiology of slider turtles is essential for understanding the evolutionary processes, conducting conservation studies, and preserving wild and exotic species (Cooke et al. 2013), as well as for the prevention and control of introduced species (Hulme 2006, Keller et al. 2008), and can also serve as a reference for areas such as animal surgery, clinic and rehabilitation (Aversi-Ferreira et al. 2005) and provide subsidies for comparative analysis with other species (Moura et al. 2007). In newly hatched *Trachemys dorbignyi*, and up to approximately two or three years of age, anatomical follow-up of the gonads is fundamental (Malvasio et al. 1999), and for *Caretta caretta*, histological assessment is also necessary (Yntema & Mrosovsky 1980 apud Malvasio et al. 1999). Secondary sexual characters such as elongated forelimb claws, long and broad tail with extended cloacal opening, and carapace edge develop rapidly during sexual maturation and are frequently used for identification of mature males (Thomas 2002, Bager 2003, Ernst & Barbour 1989 apud Vieira & Costa 2006, Readel et al. 2008), whereas in females sexual maturity is evidenced by presence of yellow vitellogenic follicles, vascularization greater than three millimeters, traces of ovulation, and eggs in the oviduct (Hamann et al. 2003, Wyneken 2004). Nevertheless, the minimum sizes indicative of sexual maturity in ocean turtles vary according to area, habitat, sex, and species (Perez et al. 2010), whereas morphophysiological changes observed in the testes, epididymides, and vas deferens are influenced by age until sexual maturity is achieved, and as well as between breeding seasons (Wyneken 2001).

The testes present fusiform morphology are bright yellowish or rosy gray, and attach to the peritoneum through the mesorchium (Lutz et al. 2003). The penis is retractable, composed by a urethral groove (sulcus spermaticus) interspersed with corpora cavernosa and located on the base of the cloaca (Wyneken 2001).

Studies addressing the biometry and morphology of *Trachemys scripta elegans* are scarce and restricted to the animals in their natural habitat, and there is no research on the reproduction anatomy of males reared in the Cerrado region. In this context, the objective of this study is to describe the biometry and morphology of the male reproductive organs of this species raised in this unique biome so that this information can be used in reproductive biology aimed at preservation actions in captivity, population control, and scientific research, in addition to enabling interspecific comparisons.

MATERIALS AND METHODS

Animals. Twenty-seven adult males of *Trachemys scripta elegans* (Wied 1839) (Testudines: Emydidae) from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, Sao Paulo state, Brazil (23°29'23.15" S; 46°31'10.90" W) (IBAMA licenses no. 136/2011 and 048/2012) were anesthetized with a combination of xylazine hydrochloride (40mg kg^{-1}) and ketamine (60mg kg^{-1}), administered intraperitoneally, and euthanized using an infusion of propofol (50mg kg^{-1}) in the spinal canal through the atlanto-occipital joint. Subsequently, the animals were frozen and sent in Styrofoam boxes containing dry ice to the Laboratory of Anatomy of Domestic and Wild Animals (LAADS) of "Universidade Federal do Vale do São Francisco" (Univasf) in Petrolina, Pernambuco state (9°23'34"S; 40°30'28"W). This study was approved by SISBIO/IBAMA (protocol no. 38601-1) and the Ethics Committee on Animal Use (CEUA) of Univasf (protocol no. 0002/160412, 0003/160412, and 0004/160412).

Biometry of secondary sexual characters and tail. At LAADS, after thawing, the following morphometric measures were taken: body mass (BM); maximum carapace length (MCL) and width (MCW); maximum plastron length (MPL) and width (MPW), and shell height (HGT); 3rd claw length (CWL), pre-cloacal tail length (PrCTL), post-cloacal tail length (PoCTL), and full tail length (FTL). BM (g) was determined using a digital precision analytical scale (Bioprecisa®, Labmais Ltda., Curitiba/PR, Brazil); MCL, MCW, MPL, MPW and HGT (cm) were measured using a millimeter measuring tape; CWL, PrCTL, PoCTL and FTL measures (cm) were taken using

a caliper with millimeter accuracy. MCL was measured in a straight line in the medial part from the anterior edge of the nuchal shield to the end of the suture between the supracaudal shields; MCW was taken as the greatest distance between the marginal shields from side to side; MPL was characterized as a measure taken from the suture between the gular scute, in the furthermost cranial part to the suture between the anal scute in the furthermost caudal part; MPW was measured from the suture between the pectoral and abdominal scute to the point where they meet the marginal shields of both antimeres; HGT was calculated by measuring the greatest distances established between the plastron and the carapace (Malvasio et al. 1999). CWL was assessed by measuring the length in a straight line from the base to the end of the third finger of the right forelimb; when this claw was worn or broken, the left antimere was evaluated. PrCTL was assessed by measuring the distance between the tail base and the cloacal opening; PoCTL was evaluated by measuring the distance between the cloacal opening and the tail end; FTL corresponded to the sum of the last two measures.

Subsequently, the specimens were sexed according to the following methodology: individuals with MCL ≥13.0cm and presence of elongated 3rd finger forelimb claw were classified as males, whereas those in which these characteristics were not observed were classified as females (Bager 2003). Sex of the study specimens was later confirmed by dissection.

Biometry and morphology of reproductive organs. The bridge structure that connects the carapace to the plastron was split using a hammer and chisel, and the plastron was removed showing the internal organs. After that, the intestines, liver, stomach, and heart were removed using fine-tip scissors, exposing the urogenital system, thus allowing sex confirmation: presence of ovaries with or without follicles and oviducts in females and presence of testes, epididymides and penis in males. The reproductive organs were removed from the coelomic cavity and submitted to biometry (weight, length, width, and thickness). The weight (g) of each organ was obtained using a digital precision analytical scale (0.001g) and the morphometric measures were taken using a caliper with millimeter accuracy. Tissue fragments of the reproductive organs were collected for routine histological processing, hematoxylin and hematoxylin-eosin staining (HE), and subsequent evaluation under light microscopy for morphological description. Sex maturation stages were identified in males by presence of spermatozoa in the seminiferous tubules and/or epididymides or by presence of differentiated spermatocytes and mature spermatids in the seminiferous tubules.

Statistical analysis. Data (mean +S.E.M.) were submitted to analysis of variance (ANOVA) with application of the *post hoc* Student's *t*-test ($p<0.05$). The Pearson correlation coefficient was used in the analysis between the assessed numerical parameters processed in Assistat 7.6 Beta® software.

RESULTS

Biometry of the body, secondary sexual characters, and tail

The following mean (minimum/maximum) values were obtained: BM = 489.27+29.84g (316.60/992.00), MCL = 15.51+0.33cm (11.90/18.90), MCW = 12.19+0.21cm (10.30/15.00), MPL = 14.11+0.27cm (12.00/15.50), MPW = 9.27+0.21cm (6.20/12/20), HGT = 5.36+0.11cm (4.10/6.50). Frequency distribution by size classes showed unimodal tendency with higher frequency of MCL at 15cm (Fig.1).

The following mean (minimum/maximum) values were also obtained: PrCTL = 1.69+0.14cm (0.70cm/3.20cm), PoCTL = 2.19+0.10cm (0.80cm/2.80cm), FTL = 4.33+0.24cm (2.00cm/5.90cm), CWL = 1.51+0.05cm (0.90cm/1.80cm).

All male individuals presented spermatozoa in smears of the testicular fluid and/or in the epididymides, and their reproductive organs could be easily identified after opening of the coelom. No statistically significant differences ($p>0.05$) were observed between right and left antimeres in the biometry of the reproductive organs (Table 1), and presented the following variations (minimum/maximum): TWe = 0.05/1.28g, TL = 0.50/1.60cm, TWi = 0.50/1.40cm, TRT = 0.10/0.60cm.

Very high correlation ($p<0.01$) was observed between the body biometric parameters, except for MCL and HGT, which presented significant correlation ($p<0.05$). Regarding the tail, significant correlation ($p<0.05$) was found between PoCTL and FTL and very significant correlation ($p<0.01$) was verified between PrCTL and FTL. CWL showed significant correlation ($p<0.05$) with BM and MPL (Table 2).

Table 3 shows the Pearson correlation (r) between the biometric parameters of the body, secondary sexual characters, and testis. Very significant correlation ($p<0.01$) was verified between BM and TWe, TL and TWi; between TWe and TL, TWi and TRT; between TL and TWi; between TWi and TRT. Significant correlation ($p<0.05$) was found between HGT and TWe, TL and TWi, and between TL and TRT.

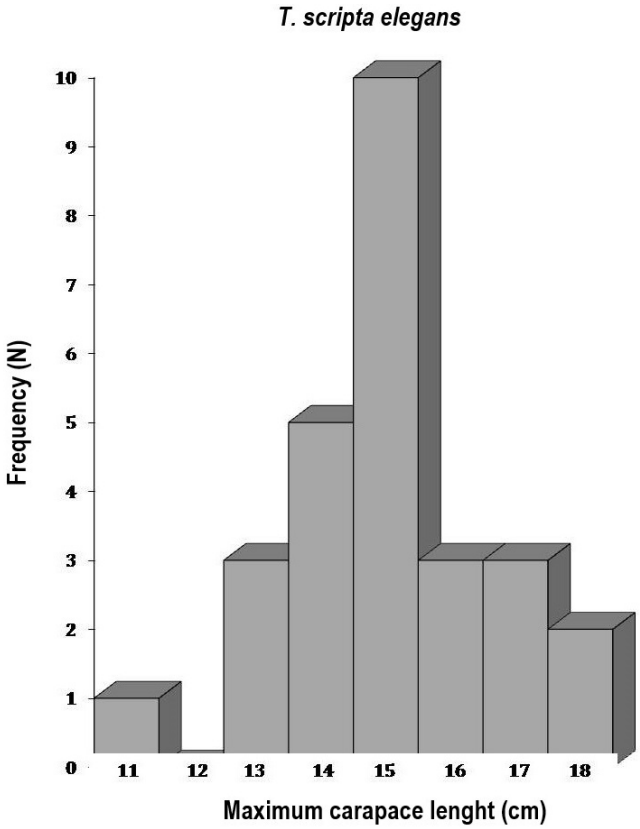


Fig.1. Frequency distribution of the maximum carapace length in males of *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. The graph shows unimodal tendency and higher frequency distribution of maximum carapace length at 15cm.

Table 1. Biometry of the reproductive organs (mean +SEM) of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil

Antimere	Weight (g)	Length (cm)	Width (cm)	Thickness (cm)
Testis				
Right	0.28 + 0.06	0.99 + 0.06	0.81 + 0.06	0.32 + 0.05
Left	0.25 + 0.06	0.97 + 0.06	0.75 + 0.05	0.26 + 0.03
Epididymis				
Right	0.58 + 0.06	2.25 + 0.11	0.76 + 0.05	0.30 + 0.04
Left	0.60 + 0.06	2.27 + 0.11	0.76 + 0.06	0.32 + 0.03
Penis	3.07 + 0.21	4.55 + 0.17	1.37 + 0.04	0.49 + 0.02

Table 2. Pearson correlation (*r*) between the biometric parameters of body and secondary sex characters of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil

	MCL	MCW	MPL	MPW	HGT	PoCTL	PrCTL	FTL	CWL
BM	0.59**	0.69**	0.73**	0.65**	0.54**	- 0.11^{ns}	- 0.07^{ns}	-0.03^{ns}	-0.47*
MCL	-------	0.79**	0.74**	0.63**	0.48*	- 0.09^{ns}	- 0.35^{ns}	- 0.24^{ns}	-0.30^{ns}
MCW	-------	-------	0.88**	0.83**	0.50**	- 0.04^{ns}	- 0.23^{ns}	- 0.18^{ns}	-0.35^{ns}
MPL	-------	-------	-------	0.83**	0.62**	- 0.10^{ns}	- 0.15^{ns}	- 0.11^{ns}	-0.38*
MPW	-------	-------	-------	-------	0.56**	- 0.14^{ns}	- 0.01^{ns}	- 0.02^{ns}	-0.48*
HGT	-------	-------	-------	-------	-------	- 0.02^{ns}	- 0.04^{ns}	- 0.11^{ns}	-0.10^{ns}
PoCTL	-------	-------	-------	-------	-------	-------	0.33^{ns}	0.46*	-0.00^{ns}
PrCTL	-------	-------	-------	-------	-------	-------	-------	0.86**	-0.11^{ns}
FTL	-------	-------	-------	-------	-------	-------	-------	-------	-0.06^{ns}
CWL	-------	-------	-------	-------	-------	-------	-------	-------	-------

* Statistically significant at 5% probability level (p<0.05), ** statistically significant at 1% probability level (p<0.01), ^{ns} not significant (p>0.05); BM = body mass, MCL = maximum carapace length, MCW = maximum carapace width, MPL = maximum plastron length, MPW = maximum plastron width, HGT =shell height, PrCTL = per-cloacal tail length, PoCTL = post-cloacal tail length, FTL = full tail length, CWL = 3rd claw length.

Table 3. Pearson correlation (*r*) between the biometric parameters of body, secondary sex characters, and testis of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil

	TWe	TL	TWi	TRT
BM	0.63**	0.53**	0.60**	0.31^{ns}
MCL	0.07^{ns}	0.08^{ns}	0.14^{ns}	0.03^{ns}
MCW	0.28^{ns}	0.33^{ns}	0.28^{ns}	0.17^{ns}
MPL	0.25^{ns}	0.26^{ns}	0.24^{ns}	0.19^{ns}
MPW	0.27^{ns}	0.25^{ns}	0.29^{ns}	0.19^{ns}
HGT	0.40*	0.40*	0.44*	0.31^{ns}
PoCTL	0.12^{ns}	0.11^{ns}	0.13^{ns}	0.11^{ns}
PrCTL	0.11^{ns}	-0.05^{ns}	0.13^{ns}	0.30^{ns}
FTL	0.08^{ns}	0.01^{ns}	0.13^{ns}	0.22^{ns}
CWL	-0.26^{ns}	-0.13^{ns}	-0.30^{ns}	-0.19^{ns}
TWe	-----	0.87**	0.96**	0.56**
TL	-----	-----	0.85**	0.45*
TWi	-----	-----	------	0.67**

* Statistically significant at 5% probability level (p<0.05), ** statistically significant at 1% probability level (p<0.01), ^{ns} not significant (p>0.05); BM = body mass, MCL = maximum carapace length, MCW = maximum carapace width, MPL = maximum plastron length, MPW = maximum plastron width, HGT =shell height, PrCTL = per-cloacal tail length, PoCTL = post-cloacal tail length, FTL = full tail length, CWL = 3rd claw length, TWe = testis weight, TL = testis length, TWi = testis width, TRT = thickness of the right testis.

No statistically significant correlation (p>0.05) was observed between the biometric parameters of body, secondary sex characters, and epididymis of male individuals except for very significant correlation (p<0.01) between EWe and EL, EWi and TRE, between EL and EWi, and between EWi and TRE (Table 4).

Regarding the penis, very significant correlation (p<0.01) was verified between BM and PWe and PL; between MCL and PWe and PL; between PoCTL and PT, and significant correlation (p<0.05) was found between PWe and MCW, MPL, HGT and CWL; between PL and MPL, HGT, PWe and PWi; between PWi and HGT (Table 5).

Gross anatomical description of the reproductive organs

The reproductive tract was located in the caudal part of the coelomic cavity, caudally to the lungs, and communicated with the external environment through the cloaca (Fig.2A). It was composed of a pair of testes, epididymides and vas deferens, and a penis (Fig.2B). The testes were located in both antimeres, were oval, bright yellowish, and positioned cranially to the epididymides and caudally to the kidneys (Fig.2A). It was not possible to identify the testicular parenchyma and mediastinum in the longitudinal section. The epididymides were located in both antimeres, consisted of delicate, grayish, convoluted tubes that elongated caudally and connected to the medium caudal end of each testis through the mesorchium that attached them to the dorsal body wall (Fig.2A,B). The epididymides were joined to the vas deferens,

which penetrated the cloacal region near the opening base of the urinary vesicle, more precisely in the spermatic sulcus located at the base of the penis (Fig.2B).

Table 4. Pearson correlation (*r*) between the biometric parameters of body, secondary sex characters, and epididymis of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil

	EWe	EL	EWi	TRE
BM	-0.33^{ns}	-0.32^{ns}	-0.38^{ns}	-0.16^{ns}
MCL	0.15^{ns}	0.05^{ns}	0.07^{ns}	0.02^{ns}
MCW	0.14^{ns}	0.14^{ns}	0.10^{ns}	0.18^{ns}
MPL	0.19^{ns}	0.13^{ns}	0.13^{ns}	0.11^{ns}
MPW	0.12^{ns}	0.23^{ns}	0.15^{ns}	0.05^{ns}
HGT	0.13^{ns}	0.29^{ns}	0.09^{ns}	-0.13^{ns}
PoCTL	0.04^{ns}	-0.14^{ns}	-0.01^{ns}	0.16^{ns}
PrCTL	-0.18^{ns}	-0.05^{ns}	-0.09^{ns}	-0.04^{ns}
FTL	-0.28^{ns}	-0.07^{ns}	-0.21^{ns}	-0.20^{ns}
CWL	0.28 ^{ns}	0.30^{ns}	0.22^{ns}	0.09^{ns}
EWe	-------	0.66**	0.86**	0.68**
EL	-------	-------	0.65**	0.24^{ns}
EWi	-------	-------	-------	0.50**

* Statistically significant at 5% probability level (p<0.05), ** statistically significant at 1% probability level (p<0.01), ^{ns} not significant (p>0.05); BM = body mass, MCL = maximum carapace length, MCW = maximum carapace width, MPL = maximum plastron length, MPW = maximum plastron width, HGT =shell height, PrCTL = per-cloacal tail length, PoCTL = post-cloacal tail length, FTL = full tail length, CWL = 3rd claw length, EWe = epididymis weight, EL = epididymis length, EWi = epididymis width, TRE = thickness of the right epididymis.

Table 5. Pearson correlation (*r*) between the biometric parameters of body and penis of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, Sao Paulo state, Brazil

	PWe	PL	PWi	PT
BM	0.68**	0.51**	0.31^{ns}	0.24^{ns}
MCL	0.56**	0.50**	0.22^{ns}	0.30^{ns}
MCW	0.43*	0.34^{ns}	-0.01^{ns}	0.26^{ns}
MPL	0.40*	0.42*	0.17^{ns}	0.27^{ns}
MPW	0.42*	0.30^{ns}	0.09^{ns}	0.34^{ns}
HGT	0.44*	0.39*	0.43*	0.19^{ns}
PoCTL	-0.12^{ns}	-0.15^{ns}	-0.22^{ns}	-0.53**
PrCTL	0.09^{ns}	-0.14^{ns}	0.05^{ns}	-0.09^{ns}
FTL	0.04^{ns}	-0.20^{ns}	0.07^{ns}	-0.08^{ns}
CWL	-0.46*	-0.09^{ns}	0.02^{ns}	-0.07^{ns}
PWe	------	0.44*	0.34^{ns}	0.11^{ns}
PL	------	------	0.46*	0.23^{ns}
PWi	------	------	------	0.21^{ns}

* Statistically significant at 5% probability level (p<0.05), ** statistically significant at 1% probability level (p<0.01), ^{ns} not significant (p>0.05); BM = body mass, MCL = maximum carapace length, MCW = maximum carapace width, MPL = maximum plastron length, MPW = maximum plastron width, HGT = shell height, PrCTL = per-cloacal tail length, PoCTL = post-cloacal tail length, FTL = full tail length, CWL = 3rd claw length, PWe = penis weight, PL = penis length, PWi = penis width, PT = penis thickness.

The penis was a single, retractile organ with copulatory function only, positioned longitudinally from the ventral wall of the cloaca. The ureters were short and opened directly into the cloaca, whereas the ductus deferens brought the spermatozoa to the urethral groove at the base of the penis. The organ was divided into a root that formed its base and had two structures, one on each side, involved in the processes of eversion and retraction; the body was composed of two corpora cavernosa interspersed with a spermatic sulcus and the glans or terminal portion (Fig.3).

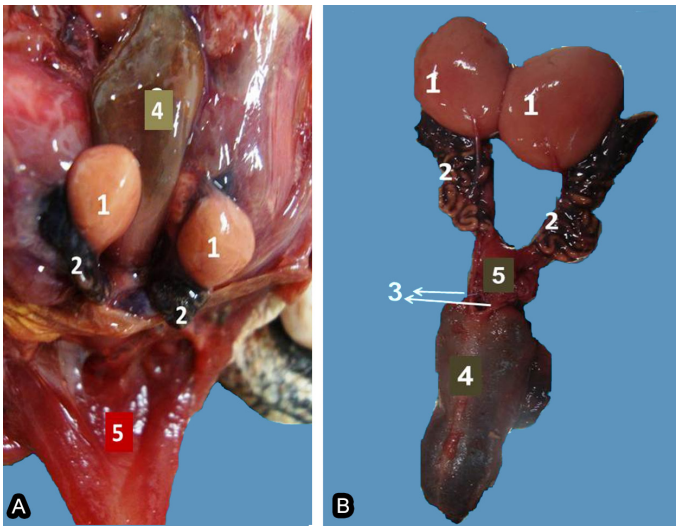


Fig.2. Reproductive tract of male *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. (**A**) View of the dorsal-caudal part of the coelom and anatomic aspect of the testes (1), epididymides (2), vas deferens - ductus deferens (3), penis (4), and cloaca (5). (**B**) Reproductive organs after removal from the coelomic cavity.

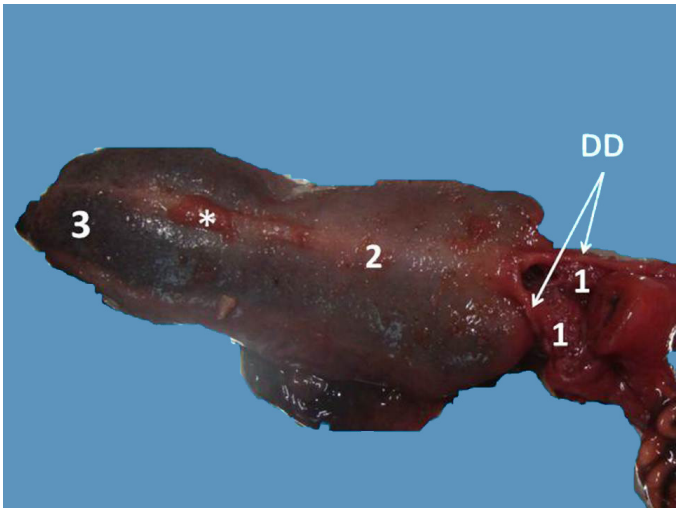


Fig.3. Penis of pubescent *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil: root (1), corpora cavernosa (2), glans (3), and urethral groove - sulcus spermaticus (*).

Morphological description of the reproductive organs

The testis was coated with a layer of dense connective tissue, rich in collagen fibers - the tunica albuginea, which provided support and protection to the organ and subdivided it into lobes filled with seminiferous tubules (Fig.4A,B), which were of varied shapes and sizes and internally lined by stratified epithelium, the seminiferous epithelium, composed of two categories of cells: support (Sertoli) and spermatogenic, with the latter represented by undifferentiated (Au) and differentiated (Ad) type A spermatogonia; primary spermatocytes at pre-leptotene (PL), leptotene (L), pachythene (PC) and zygotene (Z) stages, round spermatids (R) and spermatozoa (S) in the lumen of the seminiferous tubule (Fig.4C).

Under light microscopy, three regions of the epididymis were identified: head, body and tail. The head showed ductuli efferentes and the epididymal duct, interspersed with loose connective tissue, smooth muscle tissue, and blood vessels (Fig.5A); in the body, the epididymal duct presented a smaller diameter than that in the tail region, which had more spermatozoa. The ductuli efferentes consisted of a canalicular complex surrounded by a thin layer of smooth muscle tissue and supported by connective tissue, which emerged from a testicular network at the capitate end of the testis in number ranging from 3 to 10. The epithelium of the ductuli efferentes was of simple columnar type, and the lumen showed irregular contours and was empty (Fig.5B).

The epididymal duct was delimited by a layer of loose connective tissue interspersed with smooth muscle tissue and showed simple columnar-type epithelium composed of principal and basal cells in contact with the basal membrane (Fig.6). The principal cells were elongated, heterogeneous in shape, and contained a large amount of cytoplasm and

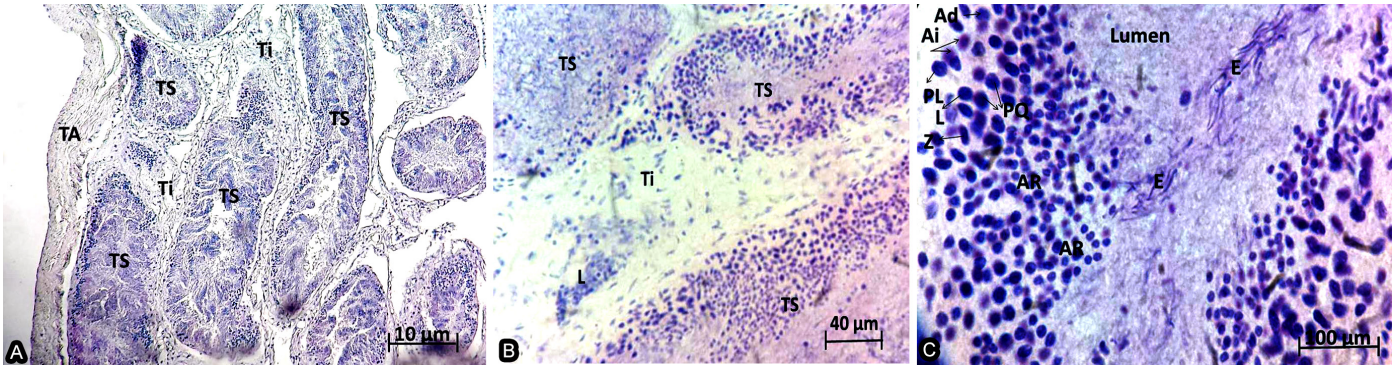


Fig.4. Testis of *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. (**A**) Tunica albuginea (TA) composed of dense connective tissue, rich in collagen fibers and seminiferous tubules (TS) surrounded by interstitial tissue (Ti) composed of loose vascular connective tissue. HE, bar=10µm. (**B**) Leydig cells (L) present in the interstitial tissue (Ti) surrounding the seminiferous tubules (TS). HE, bar=40µm. (**C**) Seminiferous epithelium at stage V of the seminiferous epithelium cycle (SEC) (Courot et al. 1970) with undifferentiated (Ai) and differentiated (Ad) type A spermatogonia; primary spermatocytes at pre-leptotene (PL), leptotene (L), zygotene (Z), and pachytene (PQ) stages; round spermatids (AR) and presence of spermatozoa (E) in the lumen of the seminiferous tubule. HE, bar=100µm.

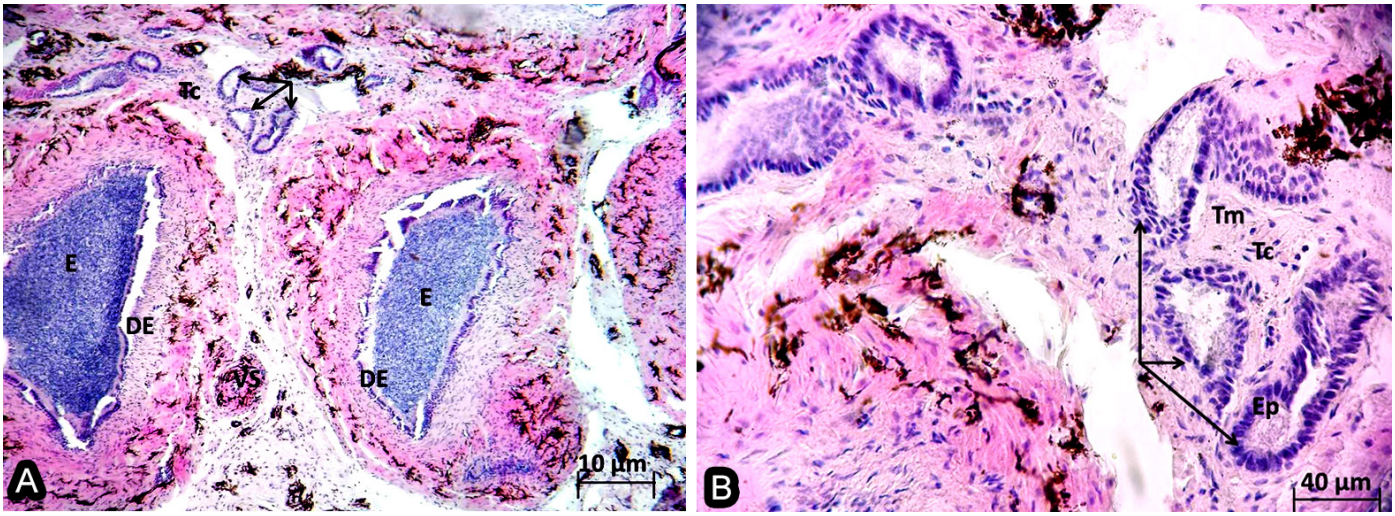


Fig.5. Epididymis of *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. Epididymal duct (DE) filled with spermatozoa (E), ductuli efferentes (arrow) and blood vessels (VS) located in the connective tissue (Tc). HE, bar=10µm. (**B**) Ductuli efferentes (arrow) surrounded by loose connective tissue (Tc) and a thin layer of smooth muscle tissue (Tm), and coated with simple columnar epithelium (Ep). HE, bar=40µm.

nucleus present in the basal pole, whereas the basal cells were flattened and located near the basal membrane of the duct, whose lumen contained a large number of spermatozoa (Fig.6). The epididymal duct continued along the body and tail of the epididymis, from which the vas deferens originated.

Histologically, the penis consisted of two corpora cavernosa around the central sulcus spermaticus (Fig.7A). The wall of the erectile body contained collagen fibers arranged in several alternate layers - the fibers were either parallel or perpendicular to the longitudinal axis of the organ (Fig.7B), and contained a groove that, during erection, formed a sulcus through which the spermatozoa was transported.

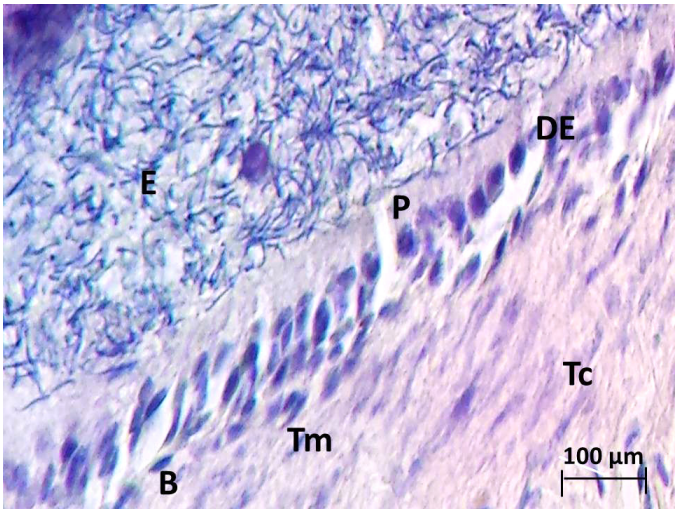


Fig.6. Epididymis of *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. Epididymal duct (DE) surrounded by simple columnar epithelium with principal (P) and basal (B) cells, surrounded by a thin layer of loose connective tissue (Tc) interspersed with smooth muscle tissue (Tm) and with presence of large number of spermatozoa (E) in its tubular lumen. HE, bar=100µm.

DISCUSSION

To the best of our knowledge, this is the first study to describe the male reproductive system of *Trachemys scripta elegans* (Testudines: Emydidae) based on biometric and morphological data. The objective was to analyze these characteristics in animals from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park and make intra- and inter-specific comparisons. It is worth noting that previous studies addressing the reproductive biology of these slider turtles have described only their reproductive behavior (Sousa et al. 2014) and the effect of temperature on sex determination (Vogt & Bull 1982, Valenzuela et al. 1997).

Mean BM values of turtle sliders from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park were similar to those of other specimens of the same origin described by Gradela et al. (2017) and of *Trachemys scripta elegans* assessed in Brasília/DF (Vieira & Costa 2006), higher than those of *Kinosternon scorpioides* (Carvalho et al. 2010), and lower than those of *Trachemys dorbigni* (Silveira et al. 2012), whereas the minimum and maximum BM values were similar to the minimum and lower than the maximum values found for *T. dorbigni* (Bager 2003). Body development occurred in a harmonious and positive way between mass, carapace, plastron and height, differing from the findings reported in the study by Gradela et al. (2017), in which uniformity in body development occurred positively only between carapace and plastron, and height was correlated only with MPL. In this study, the body biometry was slightly higher than that reported for the same species in the survey by Vieira & Costa (2006) except for MCW, lower than *T. dorbignyi* except for MPW and HGT (Silveira et al. 2012), and *T. dorbignyi* for all measures (Bager et al. 2010). The frequency of distribution by size classes was unimodal, similarly to that of *T. scripta* (Gibbons

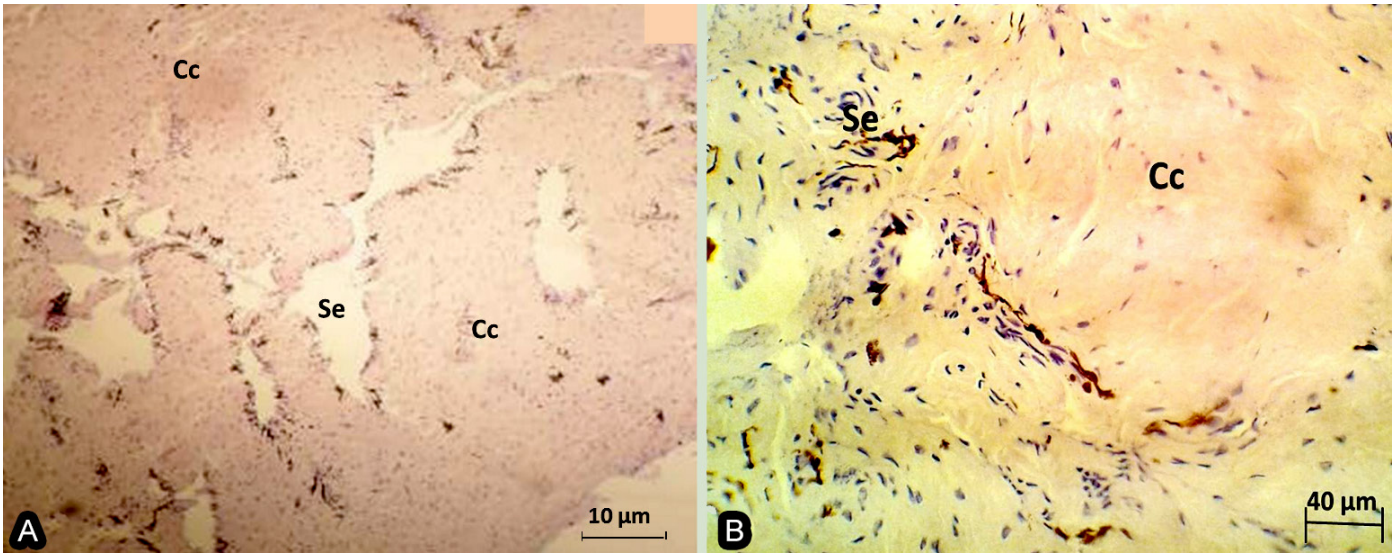


Fig.7. Penis of *Trachemys scripta elegans* from the Wild Animal Triage Center (CETA) of the Tiete Ecological Park, Guarulhos, São Paulo state, Brazil. (**A**) Corpus cavernosum (Cc) and urethral groove (sulcus spermaticus) (Se). HE, bar=10µm. (**B**) Detail of collagen fibers of corpus cavernosum and sulcus spermaticus. HE, bar=40µm.

& Greene 1990 apud Silveira et al. 2012, Lovich et al. 1990 apud Silveira et al. 2012, Gradela et al. 2017) and *T. dorbignyi* (Bager et al. 2010, Bujes et al. 2011, Silveira et al. 2012).

Secondary sexual characters, represented by elongated claws and a greater tail-cloaca distance, assist with sex identification between male and female turtles (Readel et al. 2008, Pérez et al. 2010), because the development of these characters occurs rapidly during male maturation (Gibbons & Greene 1990 apud Silveira et al. 2012, Gibbons & Lovich 1990 apud Vieira & Costa 2006, Dyce et al. 1997, Duarte et al. 2011). In addition, studies have indicated that males show greater PrCTL compared with females (Readel et al. 2008, Bujes et al. 2011, Silveira et al. 2012), which may be useful in sex identification especially in populations that vary in size according to sexual maturity, because dissection and histology of the gonads may not be feasible, particularly in the case of endangered species or for long-term studies (Readel et al. 2008). In the present study, identification of the males by the 3rd claw length was effective, corroborating the findings of studies addressing *Trachemys dorbignyi* (Thomas 2002, Bager 2003, Ernst & Barbour 1989 apud Vieira & Costa 2006, Readel et al. 2008, Cabrera 1998) and *Trachemys scripta* from Illinois, USA (Readel et al. 2008) and contrasting with the results of the research on *Trachemys adiutrix* (Batistella 2008). Regarding the measures of the tail, positive correlation was observed between FTL and PrCTL (r=0.86; p<0.01) and PoCTL (r=0.46; p<0.05), whereas very high negative correlation was found between PT and PoCTL (r=-0.53**; p<0.01).

In slider turtles male, the sexual structures were represented by a pair of testes, epididymides and vas deferens, and a penis located caudally in the coelom. These findings were similar to those reported for reptiles and vertebrates in general (Ashley 1969) and other species of turtles (Malvasio et al. 1999, Wyneken 2001, Carvalho et al. 2010). However, the absence of attached glands contrasted with mammals (Hafez & Hafez 2004), but was similar to *Kinosternon scorpioides* (Carvalho et al. 2010).

Puberty in male slider turtles begins when the testes start to produce high concentrations of testosterone, which results in the development of secondary sexual characteristics and maturity of the seminiferous tubules, with onset of spermatogenesis (Wibbels et al. 1991 apud Gomes et al. 2006). All male individuals showed presence of spermatozoa in smears of testis fluid and/or in the epididymal duct, thus being sexually mature, which seems to have contributed to the anatomical recognition of the male reproductive organs after opening the coelomic cavity, in agreement with Wyneken (2001). Wyneken observed that, in sexually mature ocean turtles, sexual dimorphism is anatomically visible with difference between ovaries and testes, whereas in *Caretta caretta* (Yntema & Mrosovsky 1980 apud Malvasio et al. 1999) and *Chelonia mydas* (Rosa 2009, Kondak 2012), gross identification of gonads is not feasible for sex identification, and histological analysis is needed. Malvasio et al. (1999) highlighted that the possibility of gross identification of gonads constitutes an advantage, because sex identification through dissection is the simplest, fastest and least expensive method in cases where external sexual characters do not show dimorphism. Although sexual maturity is dependent on the interaction between environmental and genetic factors (Stearns & Koella 1986 apud Readel et al. 2008), variation in size and maturation age may be observed within and between populations (Gibbons & Green 1990 apud Silveira et al. 2012). Studies addressing other turtle species have demonstrated that testis size and weight were correlated with MPL (Gibbons 1968 apud Readel et al. 2008, Mitchell 1985 apud Readel et al. 2008), a fact not observed in the present study.

In the testes and epididymides, the biometric similarity between antimeres contrasted with that verified in mammals, in which the left testis tends to be larger than the right one (Dyce et al. 1997), but corroborated findings in *Kinosternon scorpioides* (Carvalho et al. 2010) and *Phrynops geoffroanus* (Cabral et al. 2011a) and also contrasted with *Pelusios castaneus*, which presented differences in the length of testis and epididymis between the antimeres (Olukole et al. 2014). Only five studies on the morphometric assessment of testis in turtles were found in the literature: two were conducted with newly hatchlings (Malvasio et al. 2005, 2012), precluding comparisons with the present study because of differences in age and species; one was performed in males of *Trachemys scripta* from Illinois, USA, which were mature with CC=9.8cm, CG>0.7cm, PrCTL >1.3cm and MT >0.06g (Readel et al. 2008), similarly to the present research; two were conducted with *P. geoffroanus* (Cabral et al. 2011a) and *P. castaneus* (Olukole et al. 2014). However, none of these authors established correlations between testicular, epididymal or penile biometry and biometry of the body and secondary sexual characters. Only Rosa (2009), investigating *C. mydas,* established correlation between the measures of curvilinear carapace length and testis linear length, and verified that this correlation was low and that there was no concomitant growth in length between carapace and gonads, as observed in the present survey. Bondioli et al. (2007) associated this fact with the possible genetic variability within the same population, even in cases where there is probable common origin of the entire sample. In this study, the variables associated with carapace and plastron showed no correlation with biometry of the gonads, and correlation was only observed between biometry of the testes and BM, and HGT and among the testis parameters.

Synoptic relationship between the testes and epididymides and the kidneys was similar to that verified for *T. dorbignyi* (Malvasio et al. 1999), *K. scorpioides* (Carvalho et al. 2010), and *P. geoffroanus* (Cabral et al. 2011a), and presence of the tunica albuginea corroborated the findings on *K. scorpioides* (Carvalho et al. 2010); *P. geoffroanus* (Cabral et al. 2011a), *P. expansa*, and *P. unifilis* (Malvasio et al. 2012). Yellowish testes have been described in ocean turtles (Lutz et al. 2003), *K. scorpioides* (Carvalho et al. 2010), and other Testudines (Goulart 2004), but contrasted with whitish testes on *P. geoffroanus* (Cabral et al. 2011a), in which macroscopic differentiation between testis parenchyma and mediastinum was also not observed.

Reptilians were the first amniotes to evolve from the aquatic environment to the terrestrial environment. In order to do so, many of their reproductive characteristics needed to be changed, among them the presence of tubular testes supported by Sertoli cells and of epididymis responsible for the storage of spermatozoa (Gribbins et al. 2006). This evolution enabled the produced spermatozoa to be protected, stored and deposited safely in the female reproductive tract, rather than being released into the inhospitable terrestrial environment. The microscopic structure of the testes of the slider turtles

investigated, composed of seminiferous tubules of various sizes and shapes, with epithelium comprising spermatogenic and Sertoli cells, and interstice formed by loose connective tissue, Leydig cells, and blood vessels, was similar to that described in mammals (Junqueira & Carneiro, 2011) and other chelonians (Danni & Alho 1985 apud Malvasio et al. 1999, Wibbels et al. 1991 apud Gomes et al. 2006, Malvasio et al. 2012, Sousa et al. 2014).

Although the testes of reptiles contain seminiferous tubules filled with seminiferous epithelium, similar to those of mammals and birds, most temperate reptiles are seasonal reproducers (Gribbins et al. 2003). Therefore, germ cell development strategies in turtles are very different from that of other amniotes (Gribbins et al. 2003, Gribbins 2011), because these cells develop as the only temporary population in the testis, with a single event of spermatozoa at the end of spermatogenesis, differently from the testis of amphibians, which present continuous development of spatial germinative cells resulting in sperm release waves during mating (Russell et al. 1990 apud Gribbins et al. 2006, Kumar 1995 apud Gribbins et al. 2006). In ocean turtles, Hamann et al. (2003) reported that the spermatogenic process occurred approximately in nine months, with emergence of primary and secondary spermatocytes for six months, and then of spermatids that remained for two to three months before the maximum spermatogenesis peak. After that, the spermatozoa followed through the ductuli efferentes to the epididymides (Wyneken 2001), where they were matured and remained active for approximately 30 days (Miller 1997 apud Gomes et al. 2006). In *Pelodiscus sinensis* (Zhang et al. 2008) and *T. scripta* (Gribbins et al. 2006), spermatogenesis follows this post-nuptial pattern, that is, it begins in May with the proliferation of spermatogonia. In June, the meiotic phase begins, and continues throughout the summer and early autumn, ending with spermiation in November. This pattern of spermatogenesis development in turtles, as well as the use of the epididymides to store spermatozoa for long periods of time (Holmes & Gist 2004) so that they can be used in mating throughout the year (Gist et al. 2000), could explain the fact that, in some specimens, spermatozoa were observed only in the epididymides while the seminiferous tubules were empty.

After production inside the testes, spermatozoa are carried to the epididymides through the ductuli efferentes as described in mammals (Hafez & Hafez 2004) and other turtle species (Carvalho et al. 2010, Cabral et al. 2011a, Olukole et al. 2014). The anatomical description of the epididymides as convoluted tubules, elongated caudally, and attached to the mid-end caudal of each testis by the mesorchium, through which they attached to the dorsal wall of the body, was similar to those provided by other authors (Carvalho et al. 2010, Cabral et al. 2011a, Malvasio et al. 2012). Unlike domestic species (Dyce et al. 1997), in *T. scripta elegans*, it was not possible to differentiate anatomically between the head, body and tail of the epididymis, as in *K. scorpioides* (Carvalho et al. 2010) and *P. castaneus* (Olukole et al. 2014). Dark epididymides were also observed in *T. scripta* (Aschley 1969) and *Crysemys picta* (Holmes & Gist 2004), contrasting with their whitish color in *P. geoffroanus* (Cabral et al. 2011a) and *K. scorpioides* (Carvalho et al. 2010) and beige in *P. castaneus* (Olukole et al. 2014). After maturation in the epididymides, the spermatozoa are transported to the base of the penis through the vas deferens that deposits them in the urethral groove, as described in other species of turtles (Carvalho et al. 2010, Cabral et al. 2011a, Malvasio et al. 2012). The absence of ampulla in the ductus deferens was similar to that verified in *K. scorpioides* (Carvalho et al. 2010) and *P. geoffroanus* (Cabral et al. 2011a).

The histological morphology of the epididymis head in *T. scripta elegans*, with presence of ductuli efferentes and epididymal duct interspersed with connective tissue was similar to that of other turtles (Cabral et al. 2011b, Viana et al. 2013, Olukole et al. 2014). The ductuli efferentes are responsible for the transport of spermatozoa from the testis to the epididymis, and present epithelium capable of reabsorbing more than 90% of the luminal fluid, increasing the concentration of spermatozoa entering the epididymis (Hansen et al. 2004). In *T. scripta elegans*, groups of three to 10 ductuli efferentes were observed, as in hamsters (3-10; Ford Junior et al. 2014), mammals (5-10; Acharya et al. 2015), and *Pelusios castaneus* (5-8; Olukole et al. 2014), whereas the observed simple columnar epithelium differed from the pseudostratified columnar epithelium with areas of ciliated and non-ciliated cells in *P. geoffroanus* (Cabral et al. 2011b); of the simple cuboidal epithelium in *K. scorpioides* (Viana et al. 2013), and of the pseudostratified columnar epithelium in *Pelusios castaneus* (Olukole et al. 2014). In contrast, presence of smooth muscle tissue and loose connective tissue surrounding the ductuli efferentes, irregular contour, and empty lumen were also observed in these species. In *T. scripta elegans*, the epididymal duct showed simple columnar epithelium, contrasting with the pseudostratified columnar epithelium in *P. geoffroanus* (Cabral et al. 2011b), with epithelium varying between pseudostratified columnar and simple cuboidal in *K. scorpioides* (Viana et al. 2013), and with the pseudostratified columnar epithelium in *Pelusios castaneus* (Olukole et al. 2014). Alike to these species, the epididymal duct in *T. scripta elegans* was surrounded by smooth muscle tissue and loose connective tissue, the epithelium was composed of principal and basal cells, and the lumen was filled with spermatozoa. The heterogeneous shape of the principal cells and the location of the basal cells agreed with the findings described by Cabral et al. (2011b) and with the description in *Pelusios castaneus* (Olukole et al. 2014). In the present study, spermatozoa were found in the epididymal duct of all specimens analyzed, corroborating other studies (Viana et al. 2013, Olukole et al. 2014). According to Gist et al. (2000), in species with seasonal reproduction, spermatozoa produced during the summer are stored in the epididymides and used in mating throughout the year.

When the tetrapod evolved from the aquatic environment to the terrestrial environment, a set of traits had to evolve to enable their habitation. Thus, reproduction success in this new environment was assured by the amniotic egg, which no longer used the aquatic environment for fertilization or development, and fertilization became internal (Kardong 2011). To this end, reptiles had to develop special copulatory organs (Storer et al. 2000): lizards and snakes developed a pair of structures present laterally to the cloaca - the hemi-penis, whereas turtles and crocodiles developed a single medial phallus cranioventral to the cloaca, which seems to be homologous to the mammalian penis (Carvalho et al. 2010), developing with similarity of the embryonic origin to the gene expression pattern (Larkins &

Cohn 2015). Nevertheless, these authors pointed out that, unlike mammals, which present a closed urethral groove inside the penis, in reptiles and birds this groove remains open to facilitate the directional flow of spermatozoa along the penis, and it is termed spermatic sulcus.

Similarly to other turtle species (Wyneken 2001, Carvalho et al. 2010, Cabral et al. 2011a), the penis in *T. scripta elegans* was a single retractile organ with copulatory function only, positioned longitudinally to the ventral wall of the cloaca. As in other turtle species, it also comprised root, body and glans (Carvalho et al. 2010, Cabral et al. 2011a), and differed from the latter in mammals because they did not present penile spongy body and urethra, nor allowed the passage of urine (Hafez & Hafez 2004). The presence of structures responsible for eversion and retraction of the penis at its root and of corpora cavernosa separated by the spermatic sulcus was similar to the descriptions in *K. scorpioides* (Carvalho et al. 2010) and *P. geoffroanus* (Cabral et al. 2011a); however, unlike these, the penis of *T. scripta elegans* did not show dark and tapered glans. Goulart (2004) emphasized that the corpora cavernosa fill with blood during erection, causing distension of the penis, whereas the sulcus forms a groove through which spermatozoa is transported. Presence of collagen fibers on the wall of erectile penis, arranged in layers alternating with fibers arranged parallel or perpendicular to the longitudinal axis of the organ, also occurs in mammals and *T. scripta*, but differs from mammals because they have only one layer of fibers in each orientation (Kelly 2004); another difference is that, although the aspect that leads to the development of the penis is homologous in turtles and mammals (Raff 1996 apud Kelly 2004), the tissues that originate it are not homogeneous (Perriton et al. 2002).

CONCLUSIONS

Biometry and morphology of the reproductive tract of males of *Trachemys scripta elegans* resemble, in some aspects, those of other turtle species and mammalian species, suggesting conserved morphology among turtles and homology to mammals.

The main differences in reproductive biology occur in spermatogenesis, with development of a single germ cell population and a single event of spermatozoa at the end of it; in the long-time storage of spermatozoa in the epididymis to use in mating throughout the year; in the penis, with open urethral groove, absence of spongy body and penile urethra, and essentially copulatory function.

Histological similarity with the reproductive organs of other amniotes, including humans, can give rise to scientific and comparative studies and, together with the differences in reproductive biology, contribute to the establishment of strategies of conservation, preservation and control in reptiles.

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Development of the urinary system in guinea pig females (*Cavia porcellus*)[1]

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ABSTRACT.- Santos A.C., Lisboa-Neto A.F.S., Silva I.K.L., Pereira H.C.S., Silveira J.M., Silveira E.E., Vasconcelos B.G. & Assis-Neto A.C. 2019. **Development of the urinary system in guinea pig females (*Cavia porcellus*).** *Pesquisa Veterinária Brasileira 39(7):549-560*. Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Rua Prof. Orlando Marques de Paiva 87, São Paulo, SP 05508-270, Brazil. E-mail: antonioassis@usp.br

Guinea pigs are animal models widely used in research related to developmental biology. The objective of this work was to demonstrate the process of formation and differentiation of urinary organs in females of the species in the prenatal period. Four females were used at 25, 30, 45 and >65 DG (days of gestation). The animals were dissected, and then macroscopic and microscopic descriptions of the urinary organs were performed. At 25 DG metanephros were present in the urogenital crest into the abdominal cavity. Collecting ducts and glomerular precursor cells could be visualized. After this period, metanephros underwent microstructural modifications to form the kidneys at the end of the prenatal period. After 30 DG, the renal parenchyma already had a cortex, where the glomerulus and proximal convoluted tubules were present; and the medulla, where distal convoluted tubules, collecting ducts, and pelvis were present. The pelvis of each kidney was drained by the ureters. The ureters also underwent tissue differentiation to be differentiated (mucosa with transitional epithelium and lamina propria of connective tissue, muscular, and adventitia) at the end of the prenatal period. The urinary vesicle also underwent tissue changes to form the tunics similar to those found in the ureters, with emphasis on the greater volume of the muscular tunica and the lamina propria that constituted the submucosa in this organ. The pelvic urethra was evidenced by a mucosa lined by transitional epithelium, submucosa, muscular and adventitia. Finally, a partial clitoral urethra and a urethral meatus in the prepuce of the clitoris were also evidenced. The urethral channel began to form with the emergence of the urethral plate and the urethral groove at 30 DG and thereafter with the fusion of the urethral folds to form a partially channeled urethra in the clitoris. A urethral meatus was observed in the most distal portion of the clitoral tissue, formed by the fusion of the prepuce. It is concluded that the urinary organs of guinea pig have similar development to that described in domestic animals, except for the partial clitoral urethra and evident urethral meatus.

INDEX TERMS: Urinary system, guinea pig, *Cavia porcellus*, embryology, experimental models, metanephros, rodents.

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RESUMO.- [Desenvolvimento do sistema urinário em fêmeas de porquinhos-da-índia (*Cavia porcellus*).] Os porquinhos-da-índia são modelos animais amplamente utilizados em pesquisas relacionadas a biologia do desenvolvimento. O objetivo deste trabalho foi demonstrar o processo de formação e diferenciação dos órgãos urinários em fêmeas da espécie no período pré-natal. Foram utilizadas quatro fêmeas aos 25, 30, 45 e >65 DG (dias de gestação). Os animais foram dissecados e então, realizaram-se descrições macroscópicas e microscópicas dos órgãos urinários. Aos 25 DG os metanefros estavam presentes na crista urogenital da

cavidade abdominal. Podiam ser visualizados ductos coletores e células precursoras glomerulares. Após este período, os metanefros sofreram modificações microestruturais para formar os rins ao final do período pré-natal. Após os 30 DG, o parênquima renal já apresentava um córtex, onde estavam presentes os glomérulos e túbulos convolutos proximais, e a medula onde estavam presentes túbulos convolutos distais, ductos coletores e a pelve. A pelve de cada rim era drenada pelos ureteres. Os ureteres também sofreram diferenciação tecidual para estarem com suas túnicas diferenciadas (mucosa com epitélio de transição e lâmina própria de tecido conjuntivo; muscular; e, adventícia) ao final do período pré-natal. A vesícula urinária também passou por modificações teciduais para formar as túnicas semelhantes as dos ureteres, com destaque para o maior volume da túnica muscular e a lâmina própria que constituiu a submucosa neste órgão. Uma uretra pélvica foi evidenciada por uma mucosa revestida por epitélio de transição, submucosa, muscular e adventícia. Por último, uma uretra parcialmente clitoriana e um meato uretral no prepúcio do clitóris também foi evidenciado. O canal uretral começou a se formar com o aparecimento da placa uretral e do sulco uretral aos 30 DG e posteriormente com a fusão das pregas uretrais para formar uma uretra parcialmente canalizada no clitóris. Observou-se um meato uretral na porção mais distal do tecido clitoriano, formado pela fusão do prepúcio. Conclui-se que os órgãos urinários do porquinho-da-índia possuem desenvolvimento semelhante ao descrito em animais domésticos, com exceção da uretra parcialmente clitoriana e do meato uretral evidente.

TERMOS DE INDEXAÇÃO: Sistema urinário, fêmeas, porquinhos-da-índia, *Cavia porcellus,* embriologia, metanefros, modelos experimentais, roedores.

INTRODUCTION

The guinea pig is a hystricomorph rodent widely used as a model for researches related to reproduction and developmental biology (Stockard & Papanicolau 1919, Evans & Sack 1973, Miglino et al. 2004, Mess 2007, Oliveira et al. 2012, Vale et al. 2013, Silva et al. 2016). Clemons & Seeman (2011) described several aspects of the usefulness of the species as an experimental model. Regarding the anatomy of the species, Cooper & Schiller (1975) described the details in adults; however there is a lack of more detailed data on the urogenital apparatus of the female. On embryology, Evans & Sack (1973) described events of embryonic development, however, development of urinary and genital organs were not object of detailed study. The species is also used as pet and its pregnancy lasts about 65-68 days (Banks et al. 2010).

The urinary tract is responsible for essential functions during postnatal life. The kidneys regulate secretion of nitrogenous products, water homeostasis, electrolyte and acid-base balance, in addition to the production of hormones. The lower urinary tract regulates pressure, urine storage, and urination (Cuckow et al. 2001). The urinary organs still present many knowledge gaps, especially regarding tissue differentiation of the kidneys, after the establishment of metanephros and the formation of glomeruli and their fenestrated capillaries (Satchell & Braet 2009). Other information that needs further details is those related to the origin and formation of clitoral (in some cases partially clitoral) urethra in some females of mammals. Hyenas (*Crocuta crocuta*) were studied in the antiquity by Aristotle and continue to be studied because of the apparent masculinization of the external genitalia as demonstrated by complete channeling of the urethra in the hypertrophied clitoris of females (Yalcinkaya et al. 1993, Glickman et al. 2006, Cunha et al. 2014). The presence of a urethral ostium at the top of the clitoris in guinea pig was first described by Stockard & Papanicolau (1919). However the anatomy of the urinary organs of the species was not the main focus of the authors' study.

A peniform clitoris and a possible intersexuality were described in moles (*Talpa europaea*) (Mathews 1935). Recently, Sinclair et al. (2016) discussed the use of anatomical terminology in this type of appendage of the external genitalia in some species of mammals. For example mice and rats develop a baculum in the external genitalia, composed of bone tissue and a urethral channel to both males and females, with some minor differences (Yang et al. 2010, Weiss et al. 2012, Schlomer et al. 2013, Cunha et al. 2015, Ipulan et al. 2016). In Spix cavies (*Galea spixii*), a species phylogenetically close to the guinea pig, the tissue processes involved in the formation of the partial clitoral urethra and the urethral meatus enclosed by preputial tissue in adult females (Santos et al. 2014a, 2014b) and subsequently during intrauterine development (Santos et al. 2014c, 2018) have been defined and established.

The macroscopic process of differentiation of the external genitalia in females during embryonic development was previously established in guinea pig (Santos et al. 2016a). However the microscopic aspects and chronology of the development of the clitoral urethra were not demonstrated. Based on the importance of guinea pigs as experimental models, we developed for the first time, a study that demonstrates the process of formation and differentiation of the urinary organs of the species in the prenatal period providing data for the comparative anatomy in mammals.

MATERIALS AND METHODS

Animals. Sixteen guinea pig concepts at 25, 30, 45 and >65 (65-68) days of gestation (DG) (n=4 females per gestational period) were used. These females came from the pregnancies of 9 adult females, which were paired with 3 adult males. The study and methodology employed were duly authorized by the Bioethics Committee of the "Faculdade de Medicina Veterinária e Zootecnia" of the "Universidade de São Paulo" (Process number: 2521/2012).

Collection of conceptuses. Adult females were matched with adult males and daily monitoring of the copulation of the animals was performed by the exfoliative vaginal cytology method. Copulation was confirmed by light microscopy, through the presence of spermatozoa in the vaginal smear, in the samples transferred to microscopic slides and stained by the rapid-panoptic method. The time of detection of spermatozoa in the vaginal smear was determined as day 0 of gestation and then the time required to collect the embryos and fetuses at designated gestational ages was waited. When the corresponding gestational age reached the above-mentioned ages, the pregnant were pre-anesthetized with xylazine hydrochloride 2% (40mg/kg/IM) and ketamine hydrochloride 1% (60mg/kg/IM) and submitted to euthanasia by the administration of 2.5% sodium thiopental (60mg/kg/IV).

Dissections and macroscopic analysis. After euthanasia the females were dissected, and the embryos and fetuses were extracted from the uterus. After the female concepts were dissected, and the

urinary organs were exposed and extracted. The kidneys (left and right) of each specimen were measured using precision caliper and weighed on a precision scale. Macroscopic photo documentation was performed using Olympus SP 810UZ 14mp digital camera. Quantitative data were subjected to analysis of variance using the GraphPad InStat program and means and standard deviations were calculated. The Cramer-von Mises test for normality was used to evaluate homoscedasticity between or among variables; the Tukey comparison average test was used to assess biometric rates. Variables are unstable (CV≤15%) at significance $p<0.05$.

Light microscopy. Tissue samples were collected from the female urinary organs in each gestational age. The samples were fixed in 10% formalin solution and then dehydrated in a series of ethanol in increasing concentrations (from 50% to 100%). Then, samples were diaphanized in xylol, for later inclusion in paraffin. Cuttings of the 5μm thick from paraffin blocks were obtained in Leica RM 2155 microtome, which were stained in hematoxylin and eosin (HE). The microscopic photodocumentation was performed in an Olympus BX61VS photomicroscope. The concepts at the beginning of development (25 days) were included whole and cut respecting the following method: a cut of 5μm was used and four cuts of 5μm were discarded serially until the metanephros was found in the sample.

RESULTS

Macroscopic analysis

A pair of asymmetrical (right and left) kidneys with oval-shaped and lobate-shaped appearance kidneys was observed after 30 DG. The kidneys were in the abdominal cavity partially covered by a capsule of adipose tissue. The right kidney was positioned more cranially than the left one. Both kidneys (right and left) were associated medially with the respective adrenal glands (right and left) and the caudal vena cava. The right kidney was positioned dorsocaudally to the liver while the left kidney was positioned dorsocaudally to the greater curvature of the stomach. Each kidney had a cranial and a caudal pole, a ventral and a dorsal surface, and the medially positioned hilum, where the renal artery and vein and the ureter could be found. Left kidneys presented greater dimensions and weight when compared to right kidneys, but no statistical differences were found ($p>0.05$) (Table 1). Internally two distinct regions: cortical and medullary, in addition to the renal pelvis, positioned medially were visualized. The ureters extended caudally until they reached the urinary vesicle. The urinary vesicle had a triangular shape from 30 to 45 DG and became oval after >65 DG. The pelvic urethra continued with the urinary vesicle and was partially channeled into the clitoris (forming a urethral meatus) until it reached its external ostium at the top of the organ after 65 DG. From 30 to 45 DG, the preputial tissue of the clitoris, initially flattened, curved caudally, forming a urethral meatus (Fig.1).

Microscopic analysis

Kidneys. During intrauterine development the metanephros was circumcised by a well apparent capsule composed of dense connective tissue at 25 DG and 30 DG. After 25 DG collecting tubules were present in the cortex and medulla. After 30 DG the organ begins to differentiate into the medulla and cortex to form the kidney. The renal hilum could be found from 30 DG. Vascular structures were present in this region. The renal pelvis arose at 30 DG. It was characterized by an epithelium that varied between three types at 45 DG: prismatic pseudostratified epithelium, cylindrical pseudostratified epithelium and transitional epithelium. It had a loose connective tissue layer of the submucosa. Beneath in the mucosa were collecting tubules, segments of the nephron and vascular structures. After 30 DG, the medullary zone had segments of proximal and distal convoluted tubules and the collecting ducts. The cortical zone consisted of the efferent and afferent arteries, glomeruli and proximal and distal convoluted tubules. At 25 DG, only a cluster of precursor glomerular cells within the renal interstitium was identified. After formation of the glomerulus at 30 DG, the glomerular unit organized to form a glomerular capsule and a visible urinary space after 45 DG (Fig.2).

Ureter. At 30 DG, it was possible to observe a distinction between tunics (mucosa and lamina propria of the mucosa, muscular and adventitia). At 30 DG, the muscular layer had no characteristic muscular fibers and the submucosa had loose connective tissue. The inner lining was defined by transitional epithelium formed by several layers of cells. Its innermost surface had a plasma membrane with the presence of globular surface cells, which are responsible for forming the osmotic barrier that prevents the communication between the urine and the cellular fluids of the epithelium. It also had intermediate cells and finally the basal cells, evidenced as the smallest cells because they have a small cytoplasm, being found near the basal compartment of the luminal transitional epithelium.

At 45 DG, the histological constitution of the tunics (mucosa, lamina propria of the mucosa, muscular and adventitia) became more differentiated. It was possible to observe the lamina propria with not modeled dense connective tissue; the thickening of the muscular tunica; and, the narrowing of the adventitia. After 65 DG the tissue differentiation was even more noticeable. At this stage, the connective tissue of the lamina propria was richly vascularized. In the lower ages, connective tissue was found with little vascularization, leading to the assumption that the need for blood irrigation becomes more prominent accompanying the development of the organ. The tunica adventitia, being the outermost tunic, was in contact with the abdominal cavity and adjacent structures, such as blood vessels (Fig.3).

Table 1. Measurements of guinea pig kidneys

Measurement	Right kidneys (Mean+SD)			Left kidneys (Mean+SD)		
	30 DG	45 DG	>65 DG	30 DG	45 DG	>65 DG
Weight (g)	0.33±0.03	0.67±0.02	1.19±0.02	0.33±0.03	0.69±0.03	1.23±0.03
Length (mm)	0.47±0.02	0.78±0.04	1.02±0.05	0.47±0.04	0.81±0.04	1.04±0.03
Width (mm)	0.27±0.02	0.42±0.02	0.49±0.01	0.31±0.03	0.43±0.03	0.51±0.02
Thickness (mm)	0.24±0.03	0.39±0.01	0.48±0.03	0.25±0.03	0.42±0.03	0.50±0.03

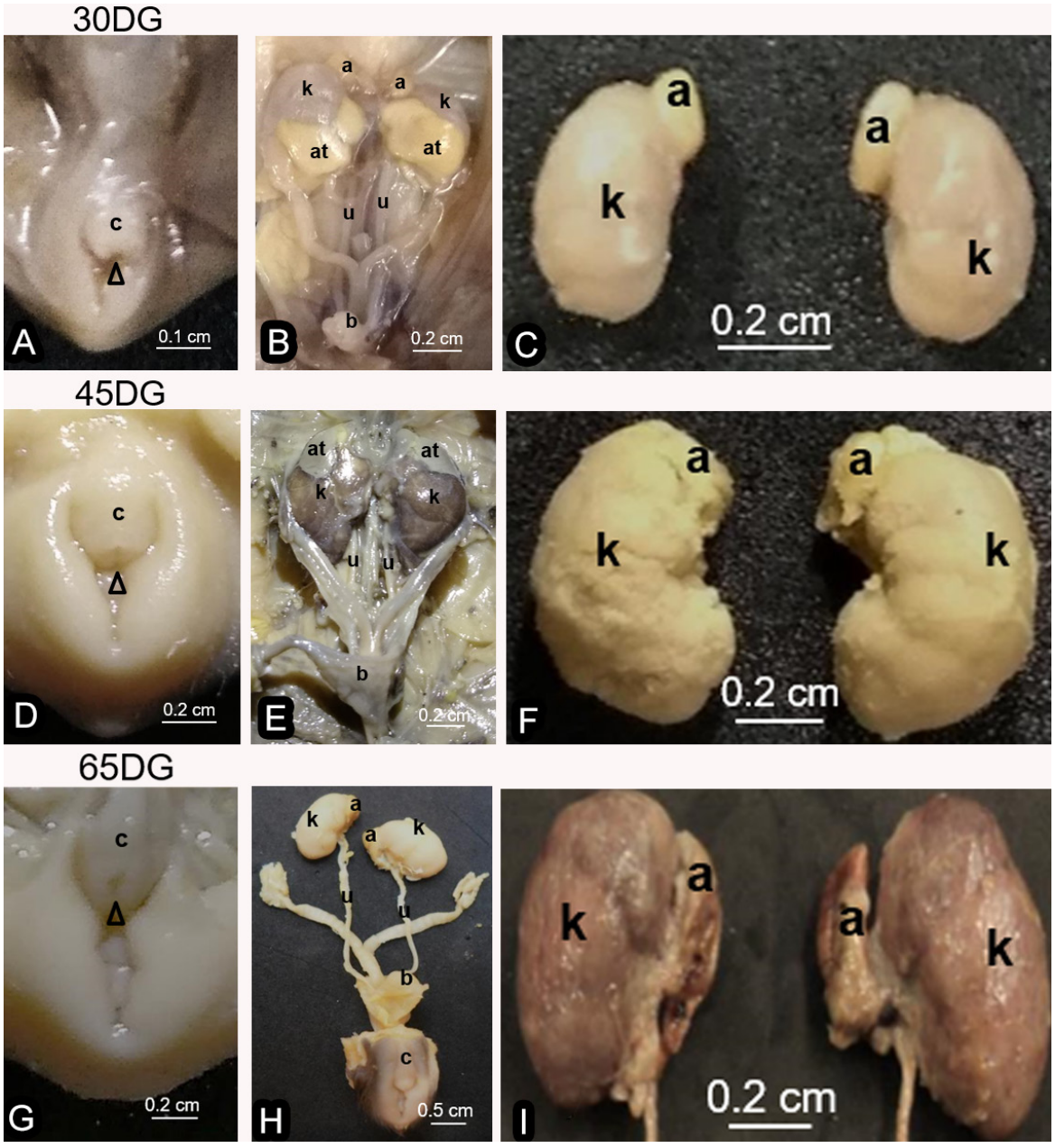


Fig.1. (**A-C**) Urinary organs development at 30 DG, (**D-F**) 45 DG and (**G-I**) >65 DG.

Urinary vesicle. The urinary vesicle presented histological characteristics like the ureter. Transitional epithelium composed of several layers of cells was present from the 30 DG until the end of gestation. The lamina propria of the mucosa formed the tunica submucosa, constituted by loose connective tissue and blood vessels, to later locate a layer of muscular tissue. The loose connective tissue of the submucosa present at 30 and 45 DG became not modeled dense connective tissue after 65 DG. The rich muscular layer increased its complexity and after the 45 DG could be observed muscular fibers longitudinally and circularly arranged. Externally, the adventitia had mesothelium with pavement cells (Fig.4).

Urethra. The urethra could be divided into two parts, a pelvic and a clitoral urethra. Clitoral urethra was characterized by a urinary channel that partially pierced the clitoris and formed a urethral meatus surrounded by preputial tissue. The pelvic portion had internal lining epithelium composing the mucosa, followed by the submucosa composed of loose connective tissue, a muscular layer and the adventitia. The mucosa was composed by transitional epithelium. The connective tissue of the submucosa at 30GD was of the loose type. At 45 DG there were regions composed of loose tissue and others composed of not modeled dense connective tissue. After the 65 DG, the connective tissue became only of the not modeled dense type with the presence of vascular elements. The muscular layer was composed of slightly defined smooth muscle between the 30 and 45 DG. After 65 DG, a rich musculature arranged with longitudinal and circular muscle bundles could be observed. The adventitial tunica that externally coated the organ was associated with the presence of blood vessels (Fig.5).

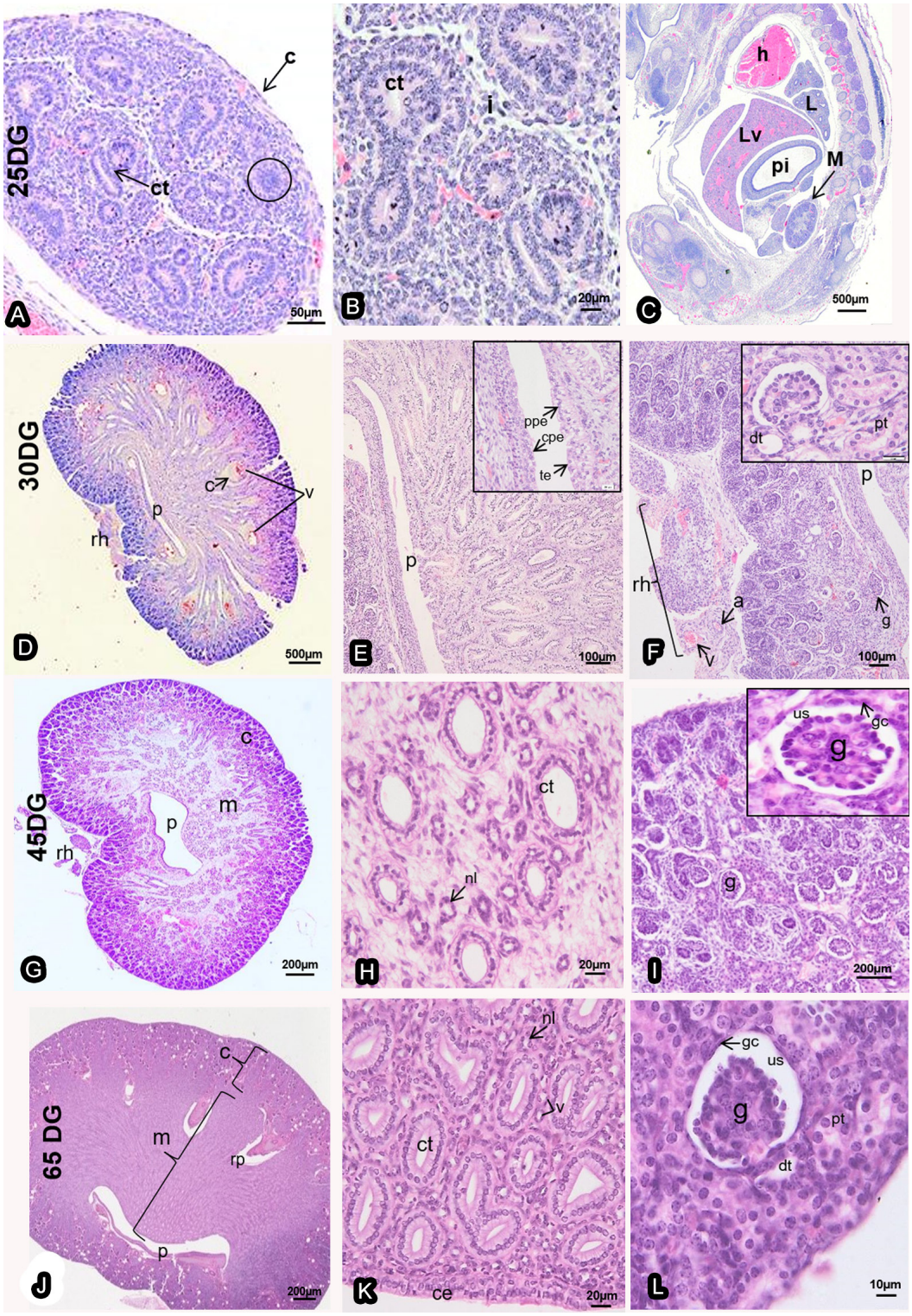


Fig.2. Development of the metanephros at (**A-C**) 25 DG and (**D-F**) of the kidneys at 30 DG, (**G-I**) 45 DG and (**J-L**) >65 DG. (**A**) Connective tissue capsule (c), collecting tubules (ct), precursor glomerular cells (circle). HE, bar = 50µm. (**B**) Renal interstitium (i), collecting tubules (ct). HE, bar = 20µm. (**C**) Metanephros (M), primitive intestine (pi), liver (Lv), lung (L), heart (h). HE, bar = 500µm. (**D**) Renal hilum (rh), pelvis (p), minor calyx (c), vessels (v). HE, bar = 500µm. (**E**) Prismatic pseudostratified epithelium (ppe), cylindrical pseudostratified epithelium (cpe), transitional epithelium (te), renal pelvis (p). HE, bar = 100µm. (**F**) Renal hilum (rh), vein (v), artery (a), glomerulus (g), distal (dt) and proximal (pt) convoluted tubule. HE, bar = 100µm. (**G**) Renal hilum (rh), pelvis (p), medulla (m), cortex (c). HE, bar = 200µm. (**H**) Collecting tubules (ct), tubular segment of the nephron (nl). HE, bar = 20µm. (**I**) Glomerulus (g), urinary space (us), glomerular capsule (gc). HE, bar = 200µm. (**J**) Cortex (c), medulla (m), renal papilla (rp), pelvis (p). HE, bar = 200µm. (**K**) Collecting tubules (ct), vessels (v), tubular segment of the nephron (nl), columnar epithelium (ce). HE, bar = 20µm. (**L**) Glomerulus (g), glomerular capsule (gc), urinary space (us), distal (dt) and proximal (pt) convoluted tubule. HE, bar = 10µm

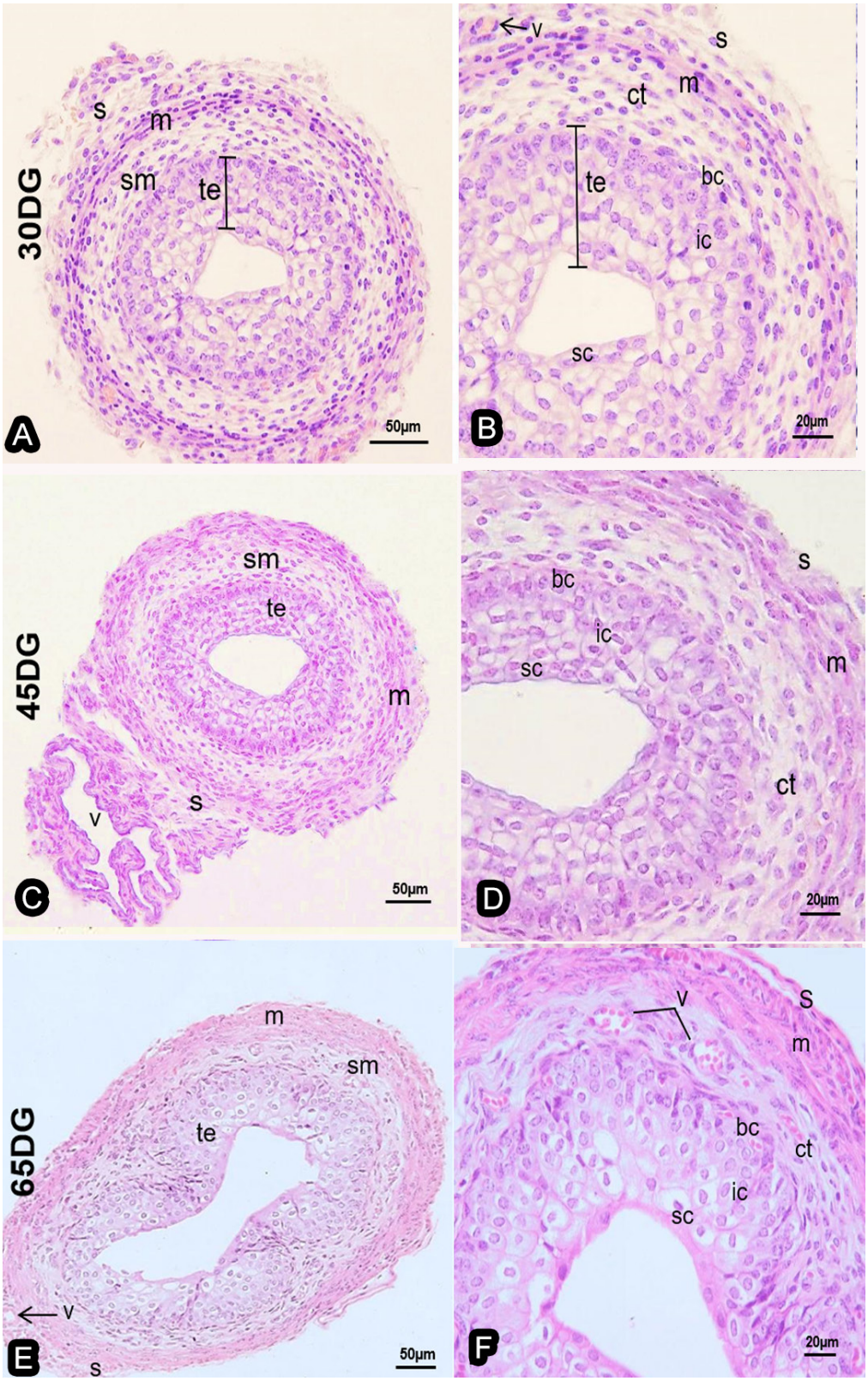


Fig.3. Development of the ureters at (**A**-**B**) 30 DG, (**C**-**D**) 45 DG and (**E**-**F**) >65 DG. (**A**) Adventitia (s), muscular layer (m), submucous (sm), transitional epithelium (te). HE, bar = 50µm. (**B**) Blood vessels (v), adventitia (s), muscular layer (m), connective tissue (ct), transitional epithelium (te) with surface (sc), intermediate (ic) and basal cells (bc). HE, bar = 20µm. (**C**) Submucous (sm), transitional epithelium (te), adventitia (s), muscular layer (m), blood vessels (v). HE, bar = 50µm. (**D**) Transitional epithelium (te) with surface (sc), intermediate (ic) and basal cells (bc), adventitia (s), muscular layer (m), connective tissue (ct). HE, bar = 20µm. (**E**) Muscular layer (m), submucous (sm), transitional epithelium (te), blood vessels (v), adventitia (s). HE, bar = 50µm. (**F**) Blood vessels (v), adventitia (s), muscular layer (m), connective tissue (ct), transitional epithelium (te) with surface (sc), intermediate (ic) and basal cells (bc). HE, bar = 20µm.

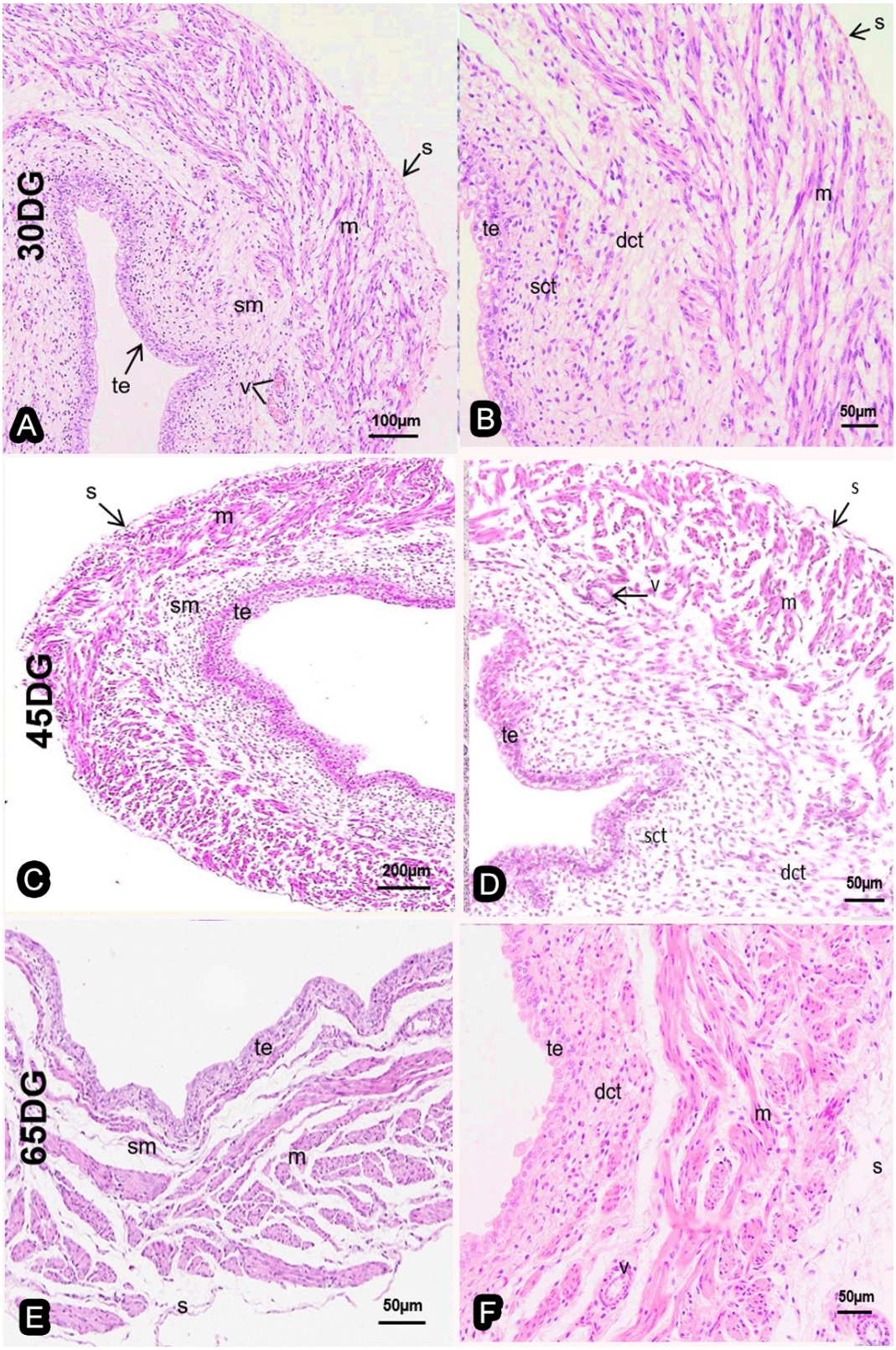


Fig.4. Development of the urinary vesicle at (**A**-**B**) 30 DG, (**C**-**D**) 45 DG and (**E**-**F**) >65 DG. (**A**) Adventitia (s), muscular layer (m), submucous (sm), blood vessels (v), transitional epithelium (te). HE, bar = 100µm. (**B**) Transitional epithelium (te), loose connective tissue (sct), not modeled dense connective tissue (dct), muscular layer (m), adventitia (s). HE, bar = 50µm. (**C**) Adventitia (s), muscular layer (m), submucous (sm), transitional epithelium (te). HE, bar = 200µm. (**D**) Transitional epithelium (te), blood vessels (v), muscular layer (m), adventitia (s), loose connective tissue (sct), not modeled dense connective tissue (dct). HE, bar = 50µm. (**E**) Submucous (sm), transitional epithelium (te), muscular layer (m), adventitia (s). HE, bar = 50µm. (**F**) Transitional epithelium (te), not modeled dense connective tissue (dct), muscular layer (m), adventitia (s), blood vessels (v). HE, bar = 50µm.

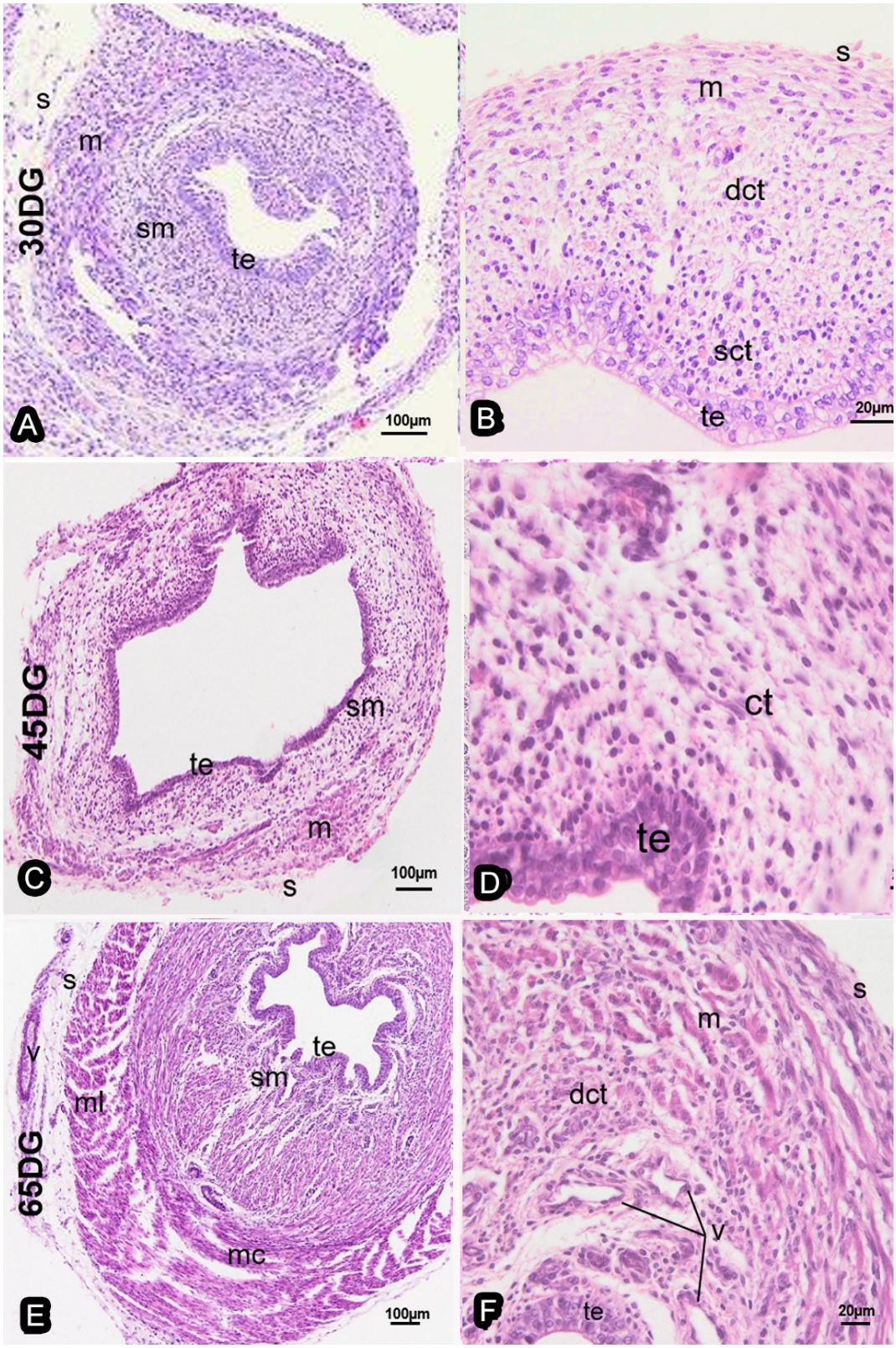


Fig.5. Development of the pelvic urethra at (**A**-**B**) 30 DG, (**C**-**D**) 45 DG and (**E**-**F**) >65 DG. (**A**) Adventitia (s), muscular layer (m), submucous (sm), transitional epithelium (te). HE, bar = 100µm. (**B**) Adventitia (s), muscular layer (m), not modeled dense connective tissue (dct), loose connective tissue (sct), transitional epithelium (te). HE, bar = 20µm. (**C**) Submucous (sm), transitional epithelium (te), muscular layer (m), adventitia (s). HE, bar = 100µm. (**D**) Submucous (sm), muscular layer (m), collecting tubules (ct), transitional epithelium (te). HE, bar = 20µm. (**E**) Adventitia (s), blood vessels (v), longitudinal muscular (ml), transitional epithelium (te), submucous (sm), circular muscular layer (mc) layer. HE, bar = 100µm. (**F**) Adventitia (s), muscular layer (m), not modeled dense connective tissue (dct), blood vessels (v), transitional epithelium (te). HE, bar = 20µm.

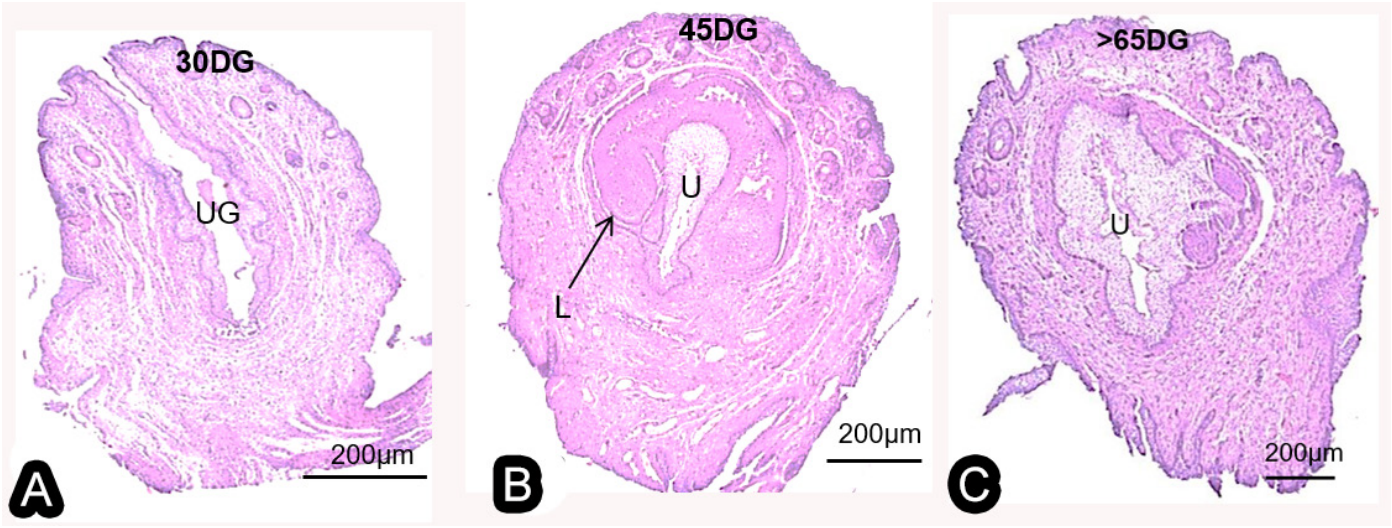


Fig.6. Development of the partial clitoral urethra and urethral meatus. (**A**) Approximation of the urethral folds and formation of the urethral sulcus (UG) at 30 DG. (**B**) Urethra (U) partially enclosed by clitoral tissue, delimited by the preputial lamina (L) at 45 DG. (**C**) Formation of the urethral meatus (U) enclosed by preputial tissue in the most distal portion of the organ after 65 DG. HE, bar = 200µm.

In the clitoral portion, the urethral mucosa was lined by transitional epithelium from its base to the apex at all ages analyzed. At 30 DG it was possible to observe the urethral plaque and the urethral sulcus without fusion of the urethral folds. At 45DG, with the fusion of the urethral folds, a urethral channel formed, being partially enclosed by clitoral tissue, which could be delimited by the preputial lamina. The preputial tissue was characteristic of the epidermis, with the presence of sebaceous glands and hair follicles, besides the external epithelial lining. It was possible to verify that, during prenatal development, the urethral glands were not found. After the 65 DG, with the elongation of the organ, a urethral meatus formed, after the end of the clitoral tissue. This meatus was surrounded by preputial tissue and opened in an ostium on the top of the organ (Fig.6).

DISCUSSION

Knowledge of the normal development of the urinary tract contributes to the understanding and perception of morphological variations between different species or even developmental anomalies (Cuckow et al. 2001, Santos Junior et al. 2014). In the present study we found that the renal development of guinea pig females, still in the intrauterine environment, presents a rapid evolution from the 25 DG until the end of gestation. From the 30 DG, the renal and conductive anatomy already had the microstructural components like that described in adults (Cooper & Schiller 1975). One detail is that the tunics have undergone tissue differentiation until the end of gestation. Highlight occurred for the fusion of the urethral folds in the genital tubercle, partially channeling the urethra into the clitoris and for the formation of a urethral meatus after the end of the clitoral tissue to open at the top of the organ. Our results demonstrated that the development of the urinary organs of the guinea pig females follow the pattern described in humans (Cuckow et al. 2001, Moore & Persaud 2008) and domestic mammals (Sinowatz 2010), except for the presence of the partially channeled urethra into the clitoris.

Chronologically the development of urinary organs in mammals involves three steps: 1) Formation and degeneration of rudimentary pronephros; 2) formation of the mesonephros which has glomerular corpuscles and degenerate completely in females, in males this transitory organ is established and modifies to form the epididymis, stimulated by androgenic hormones; 3) finally the metanephro, which originates from the ureteric bud to form the true kidney (Cuckow et al. 2001, Santos Junior et al. 2014). We did not aim to study the pronephro and mesonephro phases. In our study as described in other mammals (Cuckow et al. 2001, Sinowatz 2010), after the formation and establishment of the metanephros, these organs begin to increase their complexity with the formation of the renal pelvis and the branch of the collector ducts and by the presence of convoluted tubules. Glomeruli and portions of convoluted tubules are present in the cortex. The glomeruli begin to form in the metanephros after 25 DG by the presence of clusters of precursor glomerular cells and at 45 DG already have well defined urinary space and glomerular capsule in guinea pigs. In general, the conductive and excretory pathways of the urinary system, except for the clitoral urethra, have a similar histological conformation. That's they have mucosa with transitional epithelium and a lamina propria in the ureter, which forms a tunica submucosa in the other organs. This lamina is formed by connective tissue that passes from loose in the initial periods to not model dense in the final stages of gestation. They have a muscular layer not very evident in the initial stage of development, but that modifies forming a layer with fibers arranged circularly and longitudinally to the end of the gestation. Finally, they have an outer coating, composed of the mesothelium of the adventitial tunica, which presents blood vessels and is related to organs and structures of the abdominal cavity.

An emphasis should be given to the development of partially channeled urethra of the clitoris in the present study. This is a case where the urinary organs are closely associated with genital organs of female. This conformation

has also been investigated in other rodents of the Caviidae family, such as Spix cavies. In Spix cavies, Santos et al. (2018) demonstrated the process of fusion of the urethral folds forming a urethra, partially enclosed by the clitoral tissue. Similar to our results the urethral channeling is never complete by the clitoris; however, a urethral meatus forms after the end of the clitoral tissue and continues until it opens into an external ostium at the top of the organ. The structure that allows this morphological adaptation is a dense and fixed prepuce that continues to coat the entire clitoris like a cap, which guarantees the appearance of masculinized and hypertrophied genitalia. However through the study in Spix cavies (Santos et al. 2018) and now in the guinea pigs unlike what was thought this apparent hypertrophied clitoris channeled by the urethra is formed by preputial tissue, forming an appendix, channeled by a urethral meatus. This urethral meatus is formed after the end of the partially clitoral urethra to give continuity to the urinary channel and allow urine output to the outside.

In some rodents of the Caviidae family such as *Galea musteloides*, *Cavia aperea* (Touma et al. 2001), *Galea spixii* (Santos et al. 2016b), besides the guinea pig itself (Stockard & Papanicolau 1919, Kelly & Papanicolaou 1927), females are characterized by the formation of the vaginal closure membrane, which occludes the external vaginal ostium and part of the vulva from birth to puberty; during part of the estrous cycle, except in estrus and throughout gestation. This fact may help to explain the anatomical adaptation found since the formation of the vaginal closure membrane, external urethral ostium would be obstructed, preventing urine output to the outside. The deviation of the urethra partially to the clitoris and the formation of a preputial appendix channeled through a urethral meatus opening at the top of the organ guarantees urine output even when the vaginal closure membrane is present. It appears that this conformation follows some processes that resemble that described during the formation of the urethra in mice females, except for bone tissue that occurs in mice, but does not occur in guinea pigs (Rodriguez Junior et al. 2012, Ipulan et al. 2016). On the other hand, complete fusion of the urethra into the clitoris occurs in hyenas (Glickman et al. 2006). In hyenas this fusion resembles the process that occurs in the penis in males of the same species, which in turn resembles that described in other male mammals. In men the urethra channels the genital tubercle by the fusion of the urethral folds, being totally enveloped by the preputial lamina. In addition, another fusion occurs with the preputial tissue and suggests the "Double Zipper Hypothesis". In fact the urethra is completely channeled into the penis (Li et al. 2015). This fact did not occur with guinea pig females of the present study or even in Spix cavies (Santos et al. 2018) and members of Dasyproctidae family such as *Dasyprocta leporina* (Oliveira et al. 2017). In these species occur only the fusion of the urethral folds in the prepuce to form the urethral meatus.

The anatomical conformation of the partial clitoral urethra in guinea pigs may be better explored in future studies. A study of organ ontogeny from the formation of the undifferentiated tubercle and using specimens at shorter intervals of development could reveal more details of the morphological characterization and the adaptations necessary for the formation of the partially channeled urethra in the clitoris and the formation of the urethral meatus on the prepuce. Other studies could explore the influence of sex steroid hormones (Rodriguez Junior et al. 2012, Ipulan et al. 2016) and their respective receptors during development (Yang et al. 2010) as successfully preformed in mice. We believe that guinea pigs may be useful because they have a longer gestational period than mice and rats, which would allow extracting more details of the morphological events during the formation of the urinary organs, allowing comparisons with other mammals. It could also be useful to improve the understanding of changes resulting from malformations of these organs.

In summary the urinary organs of the species at 25 DG are constituted by metanephros, where collecting ducts and glomerular precursor cells are present. After this period metanephro undergoes microstructural modifications to form the kidneys until the end of the prenatal period, when they are divided into: cortex, where the glomeruli and proximal segments of the nephron are present; and the medulla, where distal segments of the nephron, collecting ducts, and pelvis are present. The pelvis of each kidney is drained by the ureters. The ureters also undergo tissue differentiation until the end of the prenatal period to form their differentiated tunics (mucosa with transitional epithelium and lamina propria, muscular, and adventitia). These open into the urinary vesicle. The urinary vesicle also undergoes tissue changes to form the tunics like those of the ureters, with emphasis on the lamina propria of the mucosa that becomes a tunica submucosa and due to the greater volume of the muscular tunica. The urinary vesicle is drained by the pelvic urethra, with its tunics: mucosa lined by transitional epithelium, submucosa, muscular and adventitia, which also undergo tissue differentiation. Finally, a partially clitoral urethra and a urethral meatus in the prepuce of the clitoris may also be found. This urethral channel begins to form with the appearance of the urethral plaque and the urethral groove at 30 DG and thereafter with the fusion of the urethral folds to form a partially channeled urethra at the clitoris at 45 DG. At the end of the clitoral tissue in the most distal portion, a urethral meatus formed by the fusion of the prepuce may be observed. This meatus opens into an external ostium at the top of the organ after the 45 DG.

CONCLUSIONS

The development of female guinea pig permanent urinary organs begins with the appearance and maintenance of the metanephros which undergoes tissue differentiation as the organ develops. Initially the collecting ducts and glomeruli are scattered throughout the organ's parenchyma. As the organ develops the glomeruli proliferate and migrate to the cortex. Renal pelvis formation also occurs in the late stages of prenatal development. Initially these organs have loose connective tissue that is replaced by not modeled dense connective tissue in the late stages of prenatal development.

The differentiation of muscular tunics is also evident especially in the urinary vesicle, in the late stages of intrauterine development.

The females of this species also have a partial clitoral urethra, formed by the fusion of the urethral folds, which partially channel the urethra and form a urethral meatus which in turn opens outwards in the most distal portion of the clitoris.

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