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Cover illustration: Small intestine distended by large amounts of gas of bovine with necrohemorrhagic enteritis. (Sanguanini et al., p. 698)

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Topic of General Interest

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A taste of pathology[1]

Raquel R. Rech[2], Corrie Brown[3], Jeann Leal de Araujo[4] and Claudio S.L. Barros[5]*

ABSTRACT.- Rech R.R., Brown C., Leal de Araujo J. & Barros C.S.L. 2019. **A taste of pathology**. *Pesquisa Veterinária Brasileira 39(9):673-685.* Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Müller 2443, Campo Grande, MS 79074-460, Brazil. E-mail: claudioslbarros@uol.com.br

Adult learning, or andragogy, provides a novel way of appreciating using food analogies as an effective learning tool in veterinary pathology. Facilitation of adult learning requires that new concepts be presented in a way that draws on the learner's experience. Because veterinary students will have had considerable experience with a plethora of food items prior to enrolling in a pathology course, food analogies can provide an easy conduit for incorporating key learning concepts regarding veterinary pathology. In this paper, many of these analogies are presented, along with the mechanisms responsible for each of the characteristic lesions, in the hopes that their usefulness in the classroom can be highlighted to create a more engaging and facilitated learning environment.

INDEX TERMS: Pathology, veterinary pathology, food analogies.

RESUMO.- [**Sabores da patologia.**] A aprendizagem de adultos, ou andragogia, é uma nova maneira de apreciar o uso de analogias de alimentos como uma ferramenta eficaz no aprendizado em patologia veterinária. A facilitação da aprendizagem de adultos requer que novos conceitos sejam apresentados de uma forma que se baseie na experiência do aluno. Como os estudantes de medicina veterinária já terão sido expostos a um número considerável de tipos de alimentos antes de se matricularem na disciplina de patologia, as analogias de alimentos podem fornecer um ótimo conduto para incorporar os conceitos-chave na aprendizagem da patologia veterinária. Neste artigo, muitos dessas analogias são apresentadas juntamente com os mecanismos responsáveis por cada uma das lesões características, na esperança de que sua utilidade na sala de aula possa ser destacada para criar um ambiente de aprendizado mais envolvente e favorável.

TERMOS DE INDEXAÇÃO: patologia veterinária, analogias de alimentos.

INTRODUCTION

Pathology is a central element in the teaching/learning of medicine. It is the bridge that helps students understand underlying mechanisms of disease and make the connection between organ dysfunction and clinical syndromes. If students can appreciate mechanisms of disease through judging the morphologic changes in an organ, they can better craft differential diagnoses and propose more relevant therapies. Unfortunately, the teaching of pathology often involves rote learning regarding lesions, making it difficult for students to remember each lesion and/or to grasp the significance of the morphologic changes.

Andragogy, or the science of adult learning, has received increasing attention in recent years. It is now established that adults, who includes all veterinary students globally, learn in ways that are different from children (pedagogy). One of the tenets of andragogy is that adults bring a wide range of experience into the learning environment. If adults can take a new concept and compare it to something with which they are already familiar, the filing into long-term memory is greatly facilitated (Taylor & Marienau 2016).

Although there are many who disparage the use of "kitchen pathology" as a means of learning, food analogies are widely used in human medicine (Mark et al. 2015, Vimal & Nishanthi 2017, Kobayashi & Iwasaki 2019, Sacks 2019). Indeed, when "kitchen pathology" terms are viewed in light of the science of adult learning, they make great sense and can greatly facilitate the learning of lesions. It is the purpose of this paper to review some of the prominent lesions that

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[2] Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843-4467, USA.

[3] University of Georgia College of Veterinary Medicine, Department of Pathology, 501 D.W. Brooks Drive Athens, Georgia 30602, USA.

[4] Curso de Medicina Veterinária, Universidade Federal da Paraíba (UFPB), Campus Areia, Rodovia 12, Areia, PB 58397-000, Brazil.

[5] Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Filinto Müller 2443, Campo Grande, MS 79074-460, Brazil. *Corresponding author: claudioslbarros@uol.com.br

resemble food, to give the reasons for the appearance, and to hope that they can be used successfully to allow the field of pathology to be "easier to digest".

MACROSCOPIC FINDINGS

Body cavities

Milky effusion. Chylous pleural effusion, also known as chylothorax, is defined by the presence of chyle in the pleural space. At the macroscopic level, the diagnosis of chylothorax is pursued when the appearance of the fluid is "milky" (in Portuguese: "leitoso") (Fig.1A). However, biochemical analysis is imperative to determine the type of effusion. When a chylous effusion occurs in the peritoneal cavity, it is called chyleabdomen. Chyle is a non-inflammatory, alkaline, and bacteriostatic fluid of the lymphatic system composed mainly of fat, cholesterol, electrolytes, proteins, glucose, and abundant lymphocytes, mostly T lymphocytes (Tutor 2014).

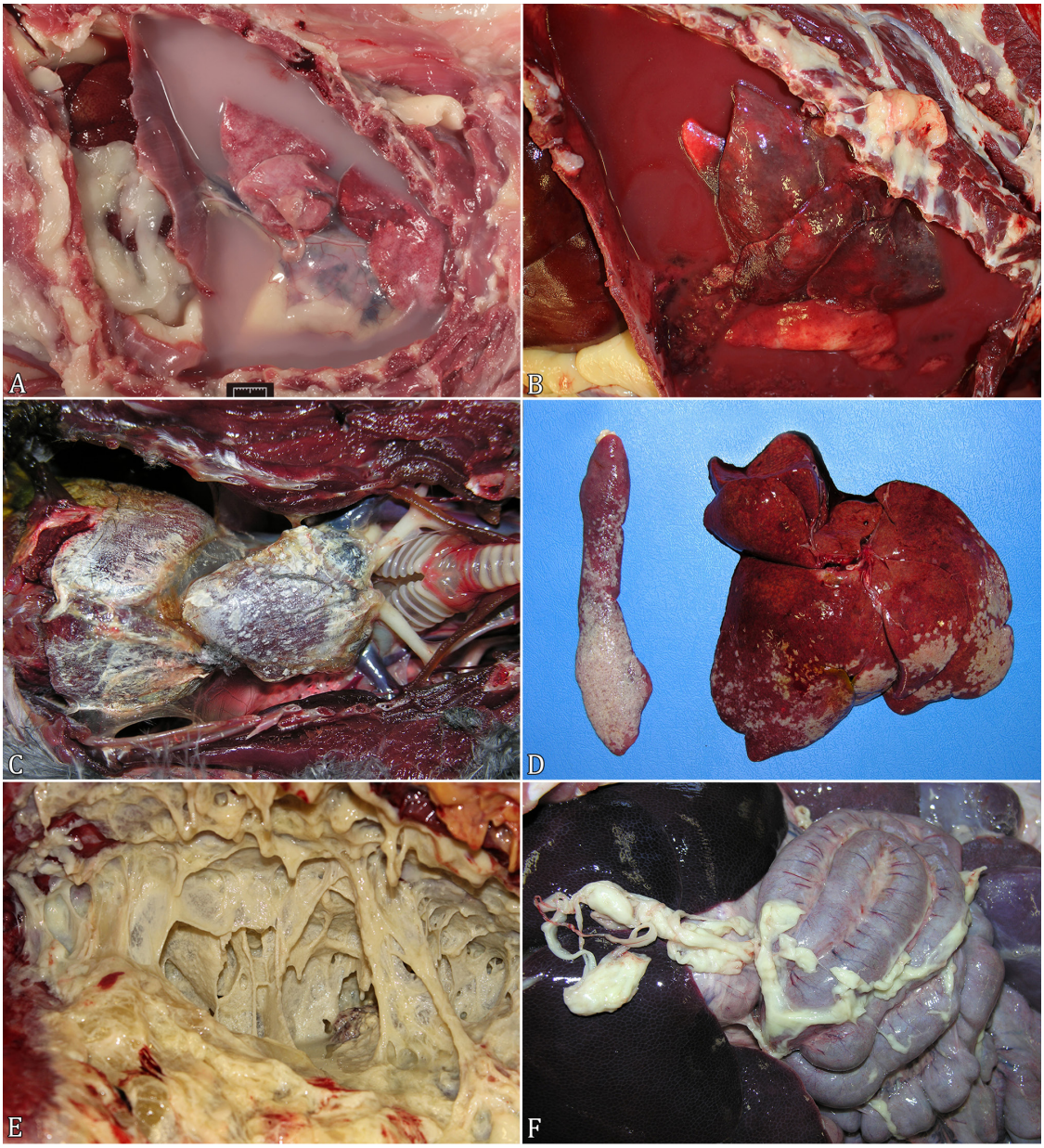


Fig.1. (**A**) Chylothorax, thorax, cat. Food analogy: milky fluid. Approximately 80mL of white, opaque fluid fills the thoracic cavity. The pressure of the accumulated fluid resulted in pulmonary atelectasis. (**B**) Marked, diffuse, chronic, pyogranulomatous pleuritis with secondary pulmonary atelectasis, thoracic cavity, dog. Food analogy: tomato soup. A thick red exudate partially fills the thoracic cavity. Note the atelectasis of the lungs. Mesothelial hyperplasia and hypertrophy is on the caudoventral area of the thoracic cavity, which it is typical in chronic inflammation of serosal surfaces. (**C**) Marked, diffuse, urate deposits; coelomic cavity; parrot; visceral gout. Food analogy: frosting or sugar icing. The heart and liver are diffusely covered by a thin layer of white, opaque, gritty material (urates). (**D**) Mild to moderate, multifocal to coalescing, chronic, fibrinous and granulomatous splenic and hepatic capsulitis, cat; feline infectious peritonitis. Food analogy: frosting or sugar icing. The splenic and hepatic capsular surfaces are multifocally covered by fibrin. (**E**) Fibrinous pleuritis, thoracic cavity, horse. Food analogy: scrambled eggs. Large accumulations of yellow, friable exudate (fibrin) loosely adhered to the parietal and visceral pleurae. (**F**) Moderate, multifocal to coalescing, acute, fibrinous peritonitis; peritoneum; pig; Glasser's disease. Food analogy: scrambled eggs. Multifocal strands of fibrin are on the surface of the liver and spiral colon.

In domestic animals, mainly dogs and cats, chylothorax occurs in cases of rupture of the thoracic duct (trauma) or, more commonly, in association with obstruction of impaired flow in the thoracic duct (López & Martinson 2017) due to regional space-occupying lesions or cardiac disease. In many cases, the cause of the chylous effusion is not found at necropsy.

Tomato soup effusion. In dogs and cats, red-brown exudate within the thoracic cavity that resembles tomato soup (in Portuguese: "sopa de tomate") (Fig.1B) is characteristic of chronic pyogranulomatous pleuritis caused by the following bacteria: *Nocardia, Actinomyces,* or *Bacteroides*. Diffuse red velvety to granular thickening of pleura reflects the fibrovascular tissue; the leakage of erythrocytes from newly formed blood vessels adds the red color to the exudate (Lopez & Martinson 2017). This exudate may contain yellowish soft granules called "sulfur granules". In chronic cases, the exudate turns brown, similar to chocolate milkshake. In dogs and cats, the disease usually occurs from inoculation of organisms through puncture wounds or foreign bodies, bites or scratches (Edwards 2006).

Sugar icing or frosting. Gout is a common metabolic disorder that results in hyperuricemia and abnormal deposition of white-chalky material on the surface of many visceral organs as well as joints of avian, reptilian and mammalian species (Fig.1C) (Lierz 2003, Fink et al. 2018). The urate aggregates have a similar appearance to frosting (in Portuguese: "glacê/cobertura de bolo"). When plasma concentration of uric acid exceeds solubility, it precipitates and accumulates on the surfaces of pericardial sac, kidney, liver, lungs, subcutaneous tissue (visceral gout) and joints (articular gout) (Lierz 2003).

Visceral gout may occur for two main reasons: reduced excretion of uric acid or increased production of uric acid (Pollock 2006). Gout is rather a clinical manifestation resulting of severe renal injury than a disease, and might be caused by toxic, infectious, nutritional or a combination of these factors. Dehydration due to water deprivation is a common factor associated with the onset of visceral gout. Nephrotoxic agents, such as mycotoxins (e.g. ochratoxin A), heavy metals (e.g. lead), anti-inflammatory drugs (e.g. diclofenac) and antibiotics (e.g. gentamycin), may also interfere with normal kidney function and consequently cause visceral gout (Shultz et al. 2004, Herbert et al. 2011). Bacterial (e.g. *Pasteurella multocida*) and viral (e.g. astrovirus, haemorrhagic polyomavirus) causes have also been implicated as potential etiologies for visceral gout (Rahimi et al. 2015, Zhang et al. 2018). On histology, urate tophi are comprised of radiating array of colorless to eosinophilic, acicular, crystalline deposits that are birefringent under polarized light.

Frosting of the surface of the abdominal organs, mainly on the surface of the liver and spleen, is observed in the wet or effusive form of feline infectious peritonitis (FIP) in cats. The disease is caused by a virulent feline coronavirus, called feline infectious peritonitis virus (FIPV), a RNA virus of the family Coronaviridae. FIPV infects and replicates in monocytes/macrophages and can gain access to the blood and induce the disease. The host determines the severity of FIP lesions. When there is no cell-mediated immune response from the host, the wet form develops. In such cases, the abdomen usually contains gelatinous, straw-colored fluid with fibrin strands both within the fluid and adhered to all serosal surfaces (fibrinous peritonitis) (Fig.1D). This arises secondary to phlebitis, mediated and dominated by activated virus-infected monocytes (Kipar & Meli 2014).

Scrambled eggs. Scrambled eggs is a dish made from eggs mixed stirred or beaten together with milk in a pan while being gently heated. Florid fibrinous exudate on the serosal surfaces, mainly in the thoracic and peritoneal cavities, resembles scrambled eggs (in Portuguese: "ovos mexidos") due to its thick, stringy, light or bright yellow appearance (Fig.1E) (Peek & McGuirk 2008). Fibrin originates from leakage of fibrinogen from capillaries and postcapillary venules secondary to severe endothelial cell injury during the acute inflammatory response.

Fibrinous exudate occurs secondarily to many bacterial infections, mainly in horses, ruminants and pigs. A few classical examples in veterinary medicine include: polyserositis in Glasser's disease caused by *Glaesserella* (*Haemophilus*) *parasuis* in pigs (Fig.1F), pleuropneumonia due to *Streptococcus equi* subsp. *zooepidemicus* in horses, traumatic reticulopericarditis (hardware disease) in cattle or any bacterial infection secondary to failure of passive immunity transfer in neonatal foals, ruminants and pigs.

Several organs

Sweetened condensed milk and cheese. The content of an abscess is usually tan to yellow, but it can widely vary in consistency. By definition, an abscess is a collection of pus (dead neutrophils) surrounded by a fibrous capsule. Neutrophils release many enzymes from their cytoplasmic granules (e.g. myeloperoxidase) to kill pyogenic bacteria such as *Staphylococcus* sp. or *Streptococcus* sp. among others. As the lesion progresses to a chronic stage, it walls off the inciting agent by producing a fibrous capsule around it (Ackermann 2017). The content of the abscesses is formed by liquefactive necrosis, called pus, and resembles sweetened condensed milk (in Portuguese: "leite condensado") (Fig.2A) or slightly creamy with a consistency of cream cheese (in Portuguese: "requeijão") (Fig.2B). In chronic abscesses or in the center of granulomas or pyogranulomas, the contents are caseous (Latin word for cheese) and become curdled with an appearance of cottage cheese (Fig.2C). Caseous necrosis occurs in the center of chronic abscesses of caseous lymphadenitis in sheep and goats and in the center of tuberculoid granulomas (Ackermann 2017).

Millet seeds. Millets are a group of highly variable small-seeded grasses, widely grown around the world as cereal crops or grains for fodder and human food. Accordingly, in descriptive pathology, the term miliary (which stems from *miliarius*, the Latin word for millet seeds; in Portuguese: "miliar") is used to describe a specific type of multifocal distribution (Stromberg 2015) in which there are numerous tiny foci randomly distributed (Fig.2D).

Alimentary system

Rice grains. Developmental stages of several parasites such as *Sarcocystis gigantea*; *Sarcocystis rileyi* and *Dipylidium caninum* resemble rice grains (in Portuguese: "grãos de arroz") on macroscopic examination. *Sarcocystis gigantea* is an Apicomplexan protozoan in the esophagus, and less commonly in the tongue, larynx and diaphragm of sheep (Fig.3A) (Dubey et al. 2015). Macrocysts remain quiescent and do not cause an inflammatory reaction to the surrounding

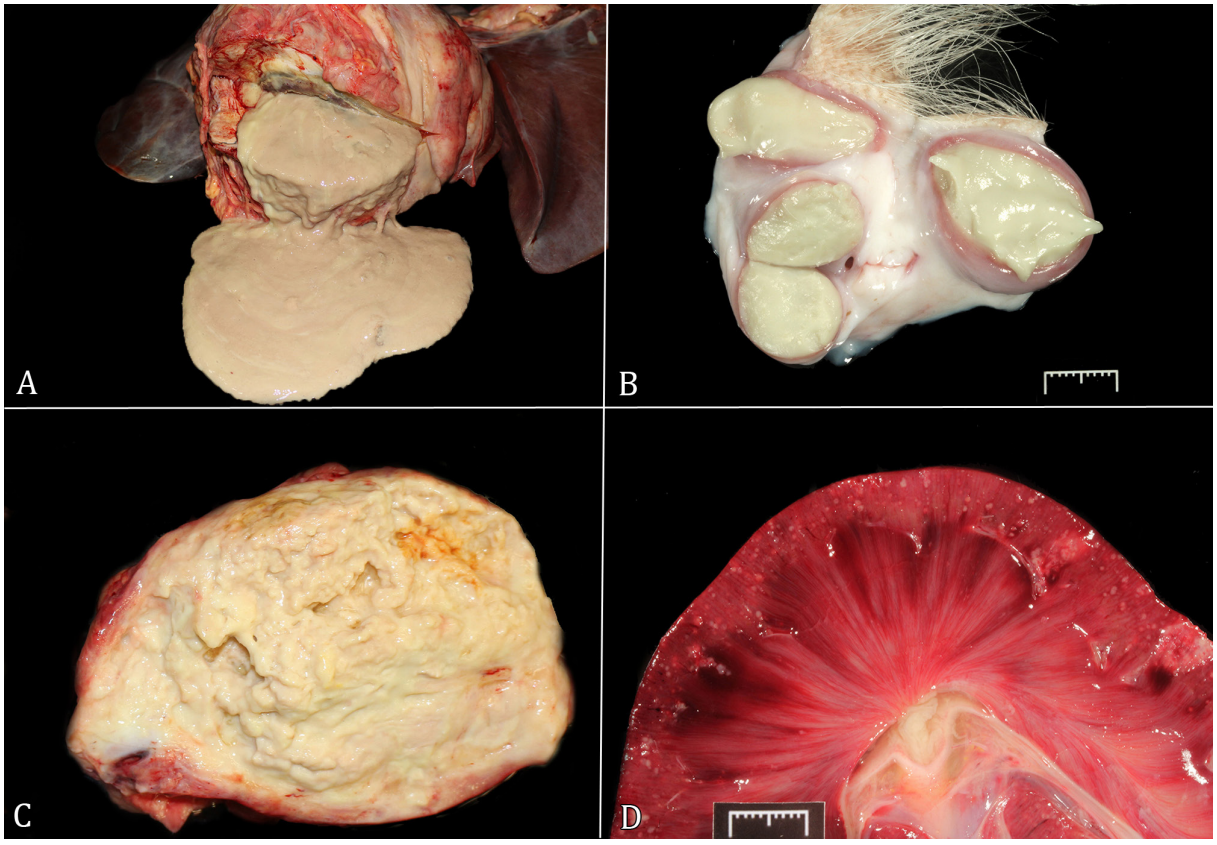


Fig.2. (**A**) Focal, chronic, hepatic abscess; liver; horse. Food analogy for the abscess content: sweetened condensed milk. On cut section, the contents are slightly yellow and liquefied (pus) surrounded by a thick fibrous capsule. (**B**) Multifocal, subcutaneous, chronic abscesses; haired skin; goat; caseous lymphadenitis. Food analogy for the abscess content: cream cheese. The contents of these abscesses are slightly yellow and creamy (pus) and are surrounded by a thin fibrous capsule. (**C**) Marked, diffuse, chronic, pyogranulomatous lymphadenitis; mesenteric lymph node; foal; *Rhodococcus equi* infection. Food analogy for the content: caseous. The mesenteric lymph node is markedly enlarged and replaced by abundant caseous material. (**D**) Moderate, multifocal, acute, suppurative embolic nephritis or glomerulitis; kidney; foal; septicemic actinobacillosis. Food analogy for the distribution of the lesions: miliary. Miliary, 0.1-0.3cm in diameter, pale tan foci are randomly distributed through the renal cortex.

musculature. Macrocysts grow for several years and bradyzoites are not highly infectious for cats, which are the definitive hosts. Although *S. gigantea* is mildly pathogenic for sheep and is not zoonotic, carcasses with cysts are still condemned during meat inspection at the slaughterhouse (Dubey et al. 2015). *Sarcocystis rileyi* occurs in the pectoral muscle of ducks and the presence of numerous macrocysts is known by the hunters as "rice breast" disease. *S. rileyi* infection in ducks occurs only in North America, which is probably related to the geographic range of the sole definitive host, the striped skunk (*Mephitis mephitis*) (Dubey et al. 2010).

Dipylidium caninum is a common tapeworm found in the small intestine of dogs. Cysticercoids develop in fleas (*Ctenocephalides canis*) and the dog acquires them by their ingestion (Bowman 2009). The flat, segmented proglottids seen in the small intestine resemble rice grains or cucumber seeds (Fig.3B).

Coffee grounds. In any species, digested blood in the stomach has a similar appearance of coffee grounds (in Portuguese: "borra de café") (Fig.3C). In domestic animals, red-brown gastric contents are usually secondary to gastric or abomasal ulcers due to a variety of causes. In dogs, causes of gastric ulcers include: (i) non-steroidal inflammatory drugs administration leading to decreased protective effects of prostaglandin E2 due to inhibition of cyclooxygenase, (ii) uremia leading to arteriolar fibrinoid necrosis and thrombosis with secondary mucosal infarction and coagulative necrosis of the gastric mucosa, (iii) paraneoplastic syndromes secondary to mast cell tumors due to increased histamine levels and gastrinomas due to increased stimulation by gastrin and elevated gastric secretion (Zollinger-Ellison syndrome). In cattle, abomasal ulcers are usually linear and can be result of stress, bovine viral diarrhea virus and malignant catarrhal fever virus or overlying the abomasal wall infiltrated by lymphoma. In calves, copper and selenium deficiency has been associated with abomasal ulcers. In pigs, the predilection site for gastric ulcers is the *pars esophagea* (Uzal et al. 2016). Nevertheless, copious nasal bleeding can be swallowed and digested blood may be present in the gastric contents. Therefore, if no gastric ulcers are noted during necropsy, the nasal cavity should be examined for potential source of bleeding.

Salt and pepper. Numerous, pinpoint, dark red foci with encysted coiled larval nematodes of small strongyles in the cecum and large colon of horses are called "salt and pepper"

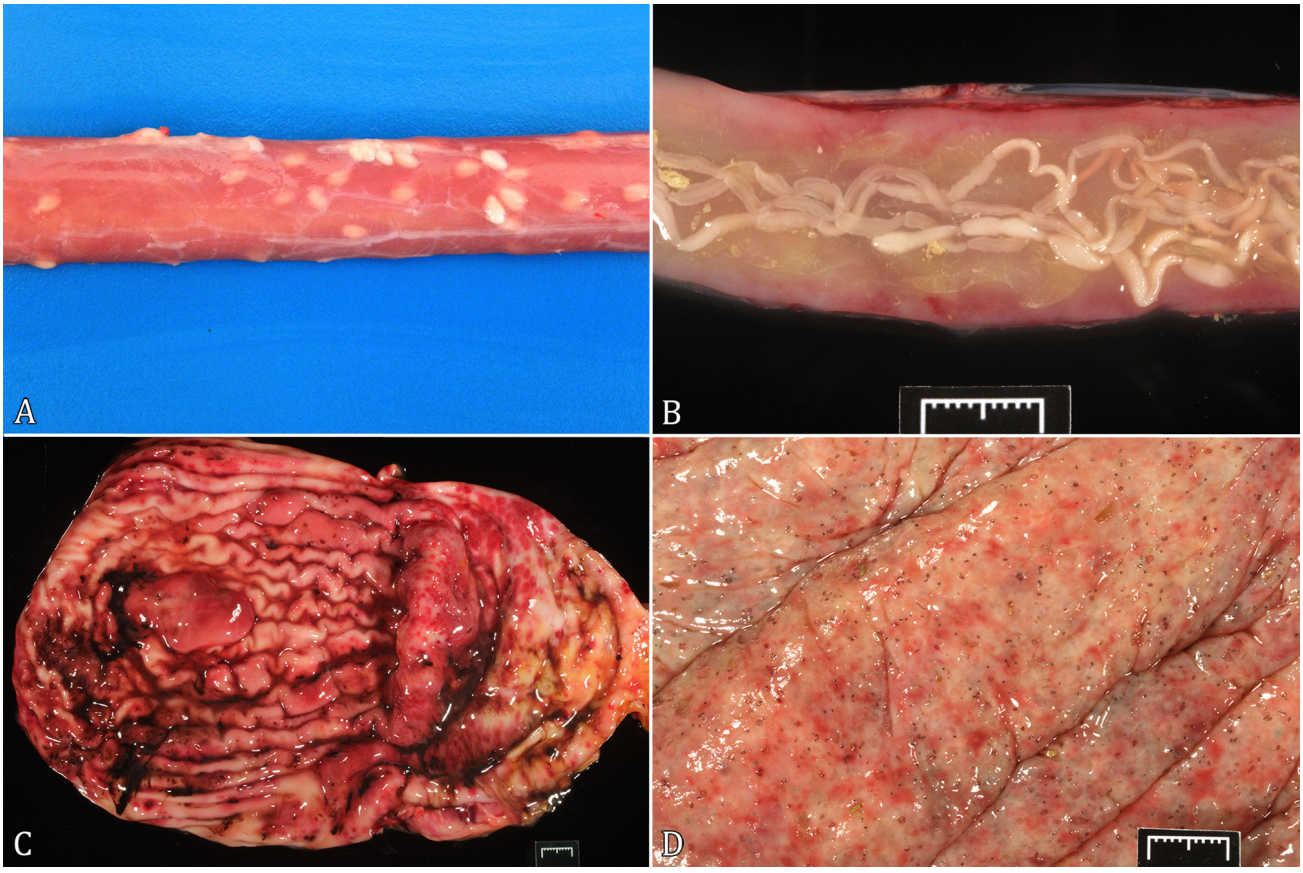


Fig.3. (**A**) Esophageal sarcocystosis; esophagus; sheep. Food analogy: rice grains. Scattered throughout the muscular layer of the esophagus are many 3-7mm long, white macrocysts of *Sarcocystis gigantea*. (**B**) Intestinal dipylidiasis; small intestine; dog. Food analogy: rice grains or cucumber seeds. The lumen of the small intestine contains numerous gravid segments of *Dipylidium caninum*. (**C**) Moderate, multifocal to coalescing, subacute, gastric ulcers; stomach, dog. Food analogy of the stomach contents: coffee ground. Multiple, linear ulcers are in the mucosa and cover by brown to black fluid (digested blood). (**D**) Marked, multifocal, chronic, granulomatous and eosinophilic colitis with mucosal and submucosal edema and cyathostomin larvae; colon, horse. Food analogy: salt and pepper colon. The colon contains numerous, 1- 5 mm long, red, coiled nematode larvae encysted in the mucosa and submucosa.

colon (in Portuguese: "sal e pimenta do reino") (Fig.3D). Small strongyles (*Nematoda, Strongylida*) are also called cyathostomes, or cyathostomins are a group of more than 50 species. They have a direct life cycle (Love et al. 1999). The infective stage is third-stage larvae where they can encyst for up to 2 years. Encysted larvae are surrounded by macrophages, lymphocytes, plasma cells and eosinophils. The emergence of arrested third-stage larvae in a short period of time causes edema and rupture of the muscularis mucosa and the development of clinical disease. Cyathostomiasis has been associated with colic and chronic diarrhea more commonly in young horses in late winter and early spring, time when the encysted larvae emerge (Uzal et al. 2016).

Hepatobiliary system

Milk spots. A common lesion in the liver of pigs is called milk spot (in Portuguese: "mancha de leite") indicating the similarity between the lesion and a drop of milk on a mahogany color surface. Milk spots are large white patches of variable and irregular contours distributed throughout all the lobes of the liver (Fig.4A). These white areas of fibrosis represent the scars caused by the migration of ascarid roundworm, *Ascaris suum,* into the liver. This lesion has no clinical significance, but a large number of organs are condemned in meat inspection; due to the aspect of milk spots the livers are rejected as unfit for human consumption (Fausto et al. 2015).

The relationship between *A. suum* larvae migration and the development of milk spots had been, for long, demonstrated experimentally (Yoshihara et al. 1983). In a nutshell, the pathogenesis is as follows: pigs ingest eggs containing third stage larvae (L3) of *A. suum* which are released in the lumen small intestine. Then, L3 migrate then through the liver parenchyma and capsule, producing necrosis initially with eosinophilic infiltrate (Nakagawa et al. 1983), which was, in the past, referred to as interstitial eosinophilic hepatitis. On resolution, the active lesions are gradually replaced by fibrous tissue, which is white and lends itself to be compared with a milk stain. Although the injury is very conspicuous in the Glisson's capsule, it can also be seen on the cut surface.

Chocolate with crisped rice. A chronic and severe hepatic lesion called end-stage liver or cirrhosis (Brown et al. 2017) is essentially a scar of the injured liver parenchyma. Typically, a dog with an end-stage liver displays loss of hepatic architecture via loss of large numbers of hepatocytes and

associated fibrosis. Regeneration of hepatocytes between bands of fibrous tissue gives rise to multiple nodules of hepatic parenchyma imprisoned by fibrosis (Fig.4B). In some Brazilian veterinary pathology laboratories, this lesion is referred to by the colorful name of a chocolate covered by caramel fudge with crisped rice (in Portuguese: "chocolate caramelizado com flocos crocantes"). The crisp aspect is somewhat similar to the nodules in the liver.

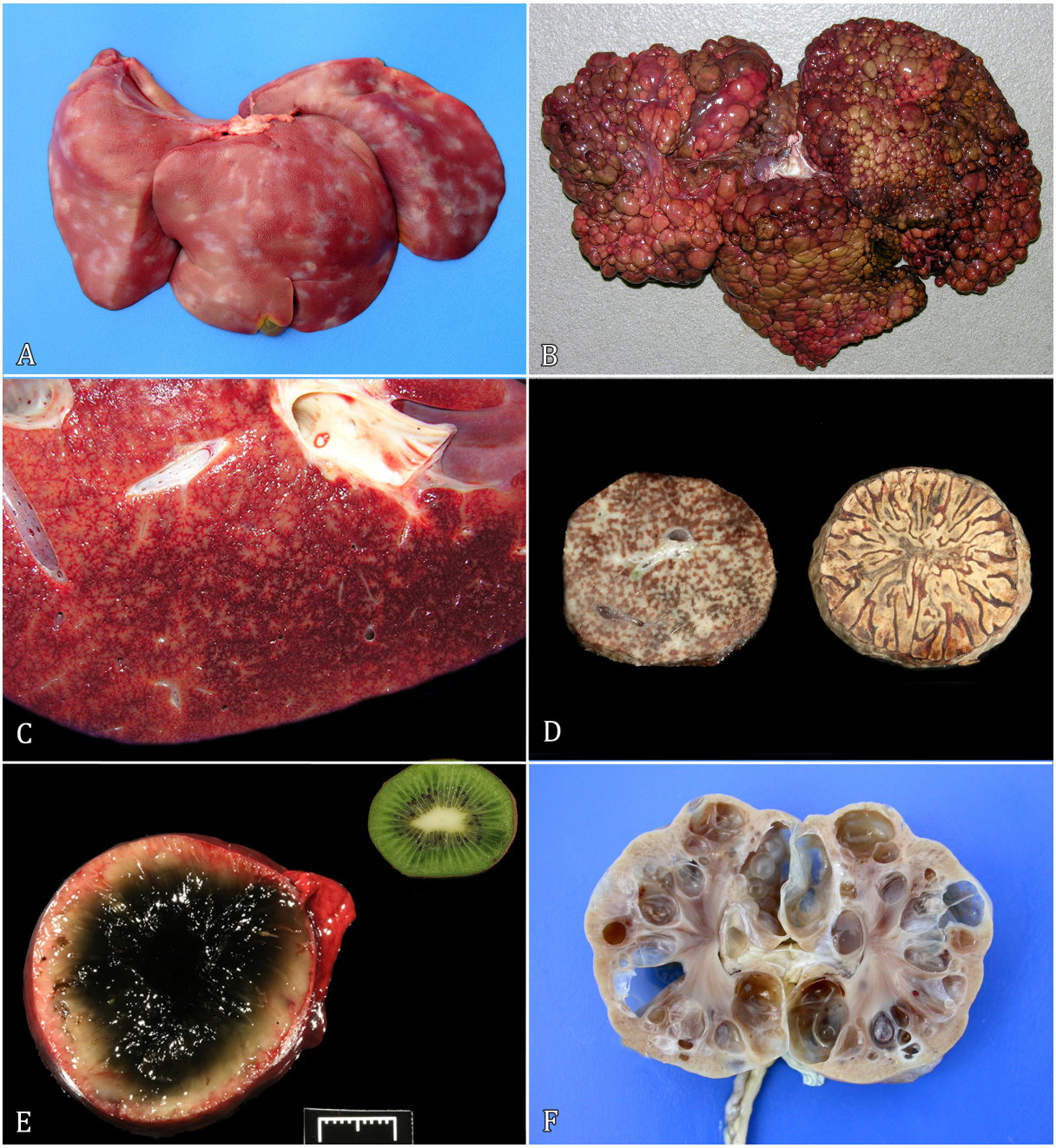


Fig.4. (**A**) Moderate, multifocal to coalescing, chronic, capsular fibrosis; liver, pig. Food analogy: milk spots. Scattered throughout the Glisson's capsule and adjacent hepatic parenchyma are multifocal to coalescing white firm areas of fibrosis. (**B**) Multinodular hepatocellular regeneration and fibrosis; liver, dog. Food analogy: chocolate with crisped rice. Nodules of attempts of hepatocellular regeneration are imprisoned (and therefore not fully functional) by bands of fibrous tissue. This lesion is also called end-stage liver or nodular hepatic cirrhosis. (**C**) Chronic congestive heart failure; liver, calf. Food analogy: nutmeg liver. Alternate areas of congestion and fibrosis in the liver imparts an aspect of nutmeg liver to the hepatic cut surface. This lesion is caused by right heart failure. (**D**) Chronic congestive heart failure; liver, calf. Food analogy: nutmeg liver. For comparison, a round fragment of the fixed liver from a dog with chronic congestive heart failure (left) is placed side by side with a piece of nutmeg of similar shape and size. (**E**) Mucocele; gallbladder; dog. Food analogy: kiwi gallbladder. On cross section, the mucosa of the gallbladder is hyperplastic and the center is filled with inspissated bile and mucus. (**F**) Multiple renal cysts; kidney, cat; polycystic kidney disease. Food analogy: swiss cheese kidney. The renal cortex and medulla are distorted by variably-sized cysts.

Nutmeg liver. Chronic congestion of the liver is almost invariably associated with congestive right heart failure (Barros 2016a). In cases of right heart failure, successive characteristic morphological changes accumulate in the liver. Blood is dammed in the center of the lobule as a result of the impediment of venous outflow from the liver usually secondary to congestive heart failure. Central lobular stasis of blood causes anoxia, hepatocellular degeneration, atrophy, and ultimately, loss of hepatocytes from the centrilobular area. Erythrocytes occupy the spaces left by the hepatocytes that went away, forming small lakes of blood. These changes are grossly observed as an accentuation of the lobular pattern, that is, red areas (blood stasis in the centrilobular areas) interspersed with lighter areas of more or less intact periportal hepatocytes. When hepatic congestion becomes chronic, there is proliferation of fibrous connective tissue filling the spaces left by the lost hepatocytes. The liver assumes a reticulated appearance due to the contrast between centrilobular zones of fibrosis, somewhat depressed and firm, alternated with areas of normal color. Over time, centrilobular fibrosis connects the centrilobular veins to each other and to the portal triads accentuating the white cross-linked tracts (Fig.4C). This change is called cardiac fibrosis due to its association with heart failure. This cross-linked pattern, especially when observed on the cut surface of the liver, resembles a nutmeg (Barros 2016a) (Fig.4D). This liver condition is, throughout the world of pathology known as nutmeg liver (in Portuguese: "fígado de noz-moscada") and occurs in all species, although it is particularly prominent in ruminants.

Some pathologists also use the term nutmeg to describe livers with centrilobular acute necrosis. However, although there is some similarity between a nutmeg and the surface of a liver with centrilobular acute necrosis, the analogy nutmeg liver should be reserved for the injury resulting from heart failure. In cases of necropsy in which nutmeg liver is observed, other indications of right heart failure such as hydrostatic edema (ascites, hydrothorax, subcutaneous dependent edema) are also seen.

Kiwi gallbladder. Mucocele is a distended gallbladder filled with excessive bile and mucus. On ultrasound examination, the accumulation of mucus in a stellate or finely striated pattern in the absence of gravity dependent bile movement is called "kiwi fruit-like" gallbladder (in Portuguese: "vesícula biliar de quivi") (Choi et al. 2014). We propose the same terminology in veterinary pathology, since the gallbladder mucocele on cross section is composed of a central area with dark green tenacious mucus and bile surrounded by a hyperplastic mucosa (Fig.4E). Gallbladder mucocele is a common cause of life-threatening biliary disease in several breeds of dogs including the Shetland Sheepdog, American Cocker Spaniel, Chihuahua, Pomeranian, Miniature Schnauzer, and Border Terriers (Allerton et al. 2018). Although the pathogenesis of the gallbladder mucocele in dogs remains unknown, an association between the mucocele and hyperadrenocorticism has been suggested (Mesich et al. 2009).

Nervous and urinary system

Swiss cheese. Swiss cheese is riddled by large holes due to fermentation. Swiss cheese appearance (in Portuguese: "queijo suíço") is characteristic of polycystic kidney disease (PKD) mainly in Persian cats (Fig.4F) (Biller et al. 1990) and in autolytic change in the brain (Rech et al. 2018). Gas-producing bacteria such as *Clostridium* spp. form multiple small cavities in many tissues, either as a result of a disease or due to postmortem proliferation.

Cardiovascular system

Bread and butter pericarditis. The non-fastidious feeding behavior of cattle results in the ingestion of foreign bodies such as metallic objects, sometimes sharply pointed, which accumulates in the reticulum. Due to the proximity of the reticulum with the heart, the gastric movements can, sometimes, push these pointed metallic objects cranially in a way that they pass through the diaphragm and penetrate the pericardium frequently puncturing the epicardium and myocardium (Peek & McGuirk 2008). Since the reticular lumen contains microbiota, the foreign body serves as a Trojan horse loaded with bacteria. An acute inflammation then ensues within the pericardial sac. Serous at its onset, the inflammation rapidly evolves to fibrinous or fibrinosuppurative pericarditis involving the external aspect of the visceral membrane (epicardium) and internal aspect of the parietal membrane (visceral pericardium) and accumulates in the pericardial sac. Several layers of fibrin are deposited in a way that when one opens the heart, the two membranes are separated, the fibrinous exudate at both membranes give the vivid impression as if two slices of bread smeared with butter were pulled apart (Fig.5A). The sobriquet bread and butter pericarditis (in Portuguese: "pão com manteiga") is then applied to describe this lesion colloquially. As time goes by, fibrovascular tissue organizes the exudate closing the space of the pericardial sac. German pathologists used to refer to this scar as *Panzer Hertz* (armored heart in English and "coração blindado" in Portuguese) in analogy to the armored tanks used in the World War II.

Mulberry heart disease. Mulberry heart disease (in Portuguese: "doença do coracão de amora") manifests by sudden death in pigs a few weeks to four months of age when the vitamin E is low. It is characterized by regions of pallor admixed with multifocal areas of hemorrhages throughout the myocardium. Mulberries are red granular elongated fruits from the trees *Morus* sp. Immature fruits are pale yellow, and turn pink while ripening to dark purple when fully ripe. Pale areas of the heart are of myocardial necrosis and the red areas resemble hemorrhage, similar to a partially ripe mulberry. The areas of hemorrhage are secondary to the fibrinoid necrosis of the arteriolar walls. Additionally, severe hydropericardium with strands of fibrin occurs secondarily to the vascular damage (Boulton 1996).

Barbiturate "salt" precipitates. An interesting artifact often occurs in animals undergoing euthanasia through barbiturates overdose. When the necropsy is carried out on these animals, the tissues previously exposed to high concentrations of these euthanasia agents, such as the endocardium of the heart and pleura following venous injection have encrusted barbiturate salt crystals (King et al. 2013, Maxie & Miller 2016). These structures are opaque white plaques (Fig.5B) that are referred to as "salt precipitates" (in Portuguese: "depósitos de sal"). Magnesium sulfate, another substance, once used as intravenous agent for euthanasia in animals has a similar effect.

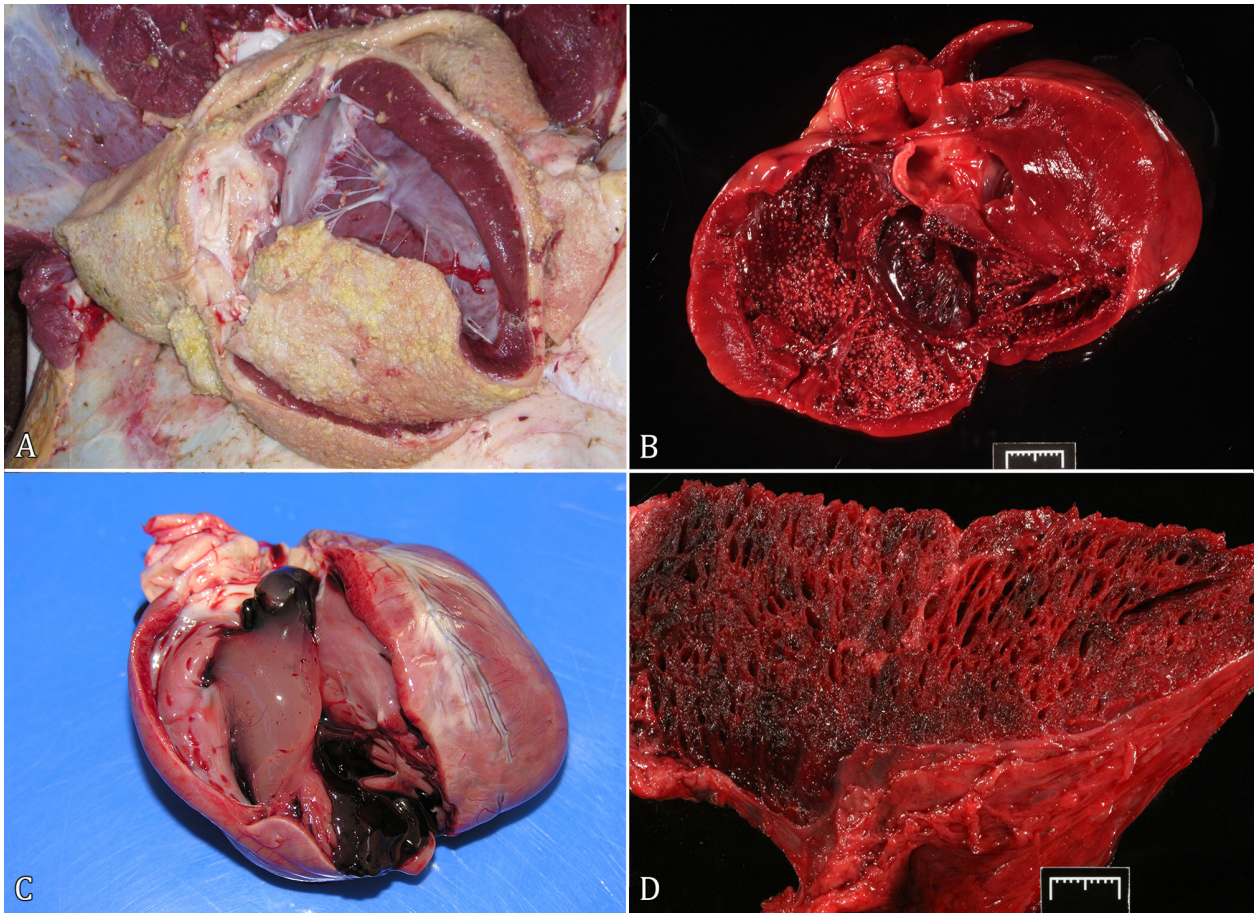


Fig.5. (**A**) Fibrinous pericarditis, pericardial sac, ox; hardware disease. Food analogy: bread and butter pericarditis. The surface of the epicardium is rough, granular and diffusely covered by fibrin. (**B**) Multifocal, marked, barbiturate salt crystals deposition; endocardium, right ventricle, dog; Food analogy: salt precipitates. The endocardium has incrusted barbiturate salt crystals that appear as opaque white plaques. (**C**) Postmortem clots, heart, dog. The right ventricle contains a pale yellow clot (Food analogy: chicken fat) and dark red clots (Food analogy: currant jelly). (**D**) Diffuse, marked, acute, necrohemorrhagic and emphysematous myositis; skeletal muscle (hindquarter); heifer. Food analogy: aerated chocolate. The skeletal muscle contains multifocal to coalescing areas of hemorrhage and necrosis admixed with many air bubbles cavities (emphysema). Reproduced with permission from Abreu et al. 2017.

Currant jelly and chicken fat clots. When an animal dies, shiny and smooth *postmortem* clots form within the right ventricle and atrium as well as in the large vessels. Postmortem clots conform to the shape of the heart chamber (Fig.5C) or vessel in which they form. Red clots are usually at the bottom of the clot and contain sedimented erythrocytes. They are called "currant jelly" (in Portuguese: "geleia de groselha"). The yellow portion represents clotted plasma devoided of red blood cells and is referred to as "chicken fat" (in Portuguese: "gordura de galinha"). Horses more often have pale clots because of a rapid erythrocyte sedimentation rate termed rouleaux formation (Myers et al. 2012). At necropsy, it is important to differentiate *postmortem* clots from *antemortem* thrombi. Thrombi have a dull surface and are adherent to the vessel wall.

Musculoskeletal system

Aerated chocolate. Necrohemorrhagic and emphysematous myositis and myocarditis are characteristic lesions caused by *Clostridium chauvoei* infection. Due to hemorrhage and necrosis, the affected muscles of the limbs turn dark-red; therefore the disease is called blackleg (Abreu et al. 2017). The emphysematous (gas bubbles) black (necrotic) areas of musclehave the appearance of aerated chocolate (in Portuguese: "chocolate aerado") on cut surface (Fig.5D).

After death, the process of decomposition of a body, involves autolysis and bacterial putrefaction. As a result of bacterial putrefaction, body cavities and organs become distended by gas bubbles (Brooks 2016) which along with the discoloration of the autolyzed tissues, as the liver, also impart the appearance of aerated chocolate (Rech et al. 2013).

Hematopoietic

Cola colored urine. Animals with intravascular hemolytic disease or degenerative myopathy present dark urine (Fig.6A), which pathologists refer to as "cola-colored urine" (in Portuguese: "urina cor de refrigerante de cola") due respectively to the presence of the dark pigment of hemoglobin or myoglobin in the urine. The analogy is with the substance extracted from the nut-cola (the fruit of plants of the genus *Cola*) that was used to produce "cola" soft drinks and conferred the coffee color characteristic of these beverages. In intravascular

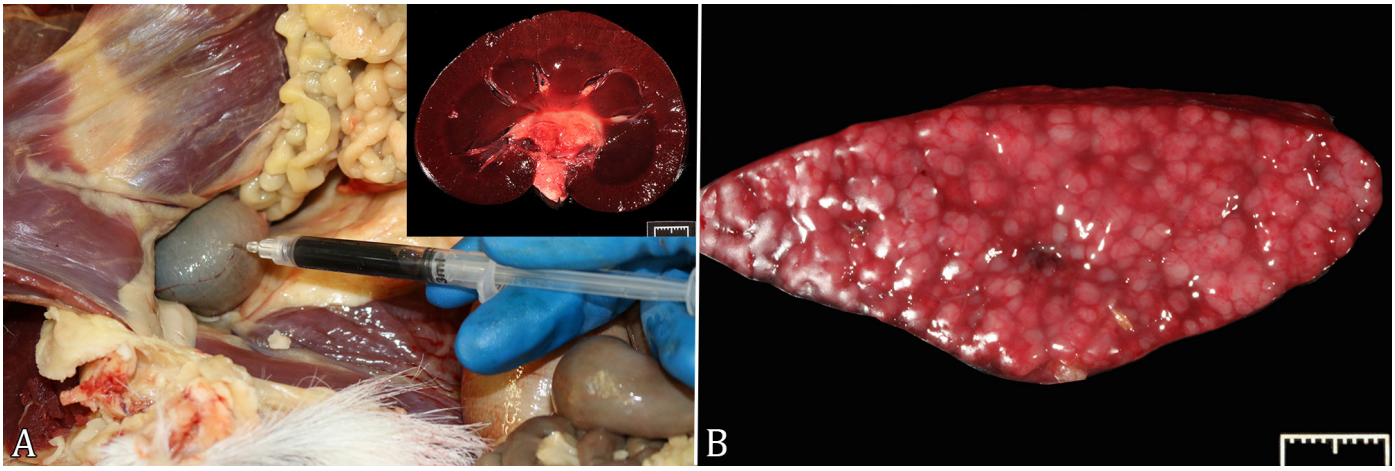


Fig.6. (**A**) Hemoglobinuria; urinary bladder, sheep; copper toxicosis. Food analogy: cola colored urine. The urinary bladder contains dark red urine. The adipose tissue is moderately yellow (icterus). Inset: hemoglobinuric nephrosis; kidney, sheep. The renal cortex and medulla are diffusely dark red due to acute tubular necrosis with intratubular hemoglobinuric casts. (**B**) Marked, multifocal, lymphoid follicular hyperplasia, spleen, dog. Food analogy: sago spleen. Traditionally sago spleen is the term for splenic follicular amyloidosis. However, some pathologists by analogy refer to marked lymphoid hyperplasia as sago spleen. Image courtesy of Dr. Laura Bryan.

hemolysis, the destruction of erythrocytes releases large amounts of hemoglobin in the circulation causing marked hemoglobinemia. The hemoglobin passes through the kidney and is collected within the urinary bladder admixed with urine, imparting the dark-red color.

Animals with intravascular hemolytic disease present jaundice, dark kidneys (due to hemoglobin impregnation) and hemoglobin imbibition of the serosa of the viscera. The spleen is enlarged with a tense capsule. Examples of this disorder occur in all domestic species. In cattle, it occurs in babesiosis (Barros et al. 2006), bacillary hemoglobinuria (Barros 2016a) and leptospirosis in calves up to approximately 30 days old. In sheep, it is observed mainly in chronic copper intoxication (Pilati et al. 1990). In dogs, it is associated with babesiosis, immune-mediated hemolytic diseases and as a complication of hemolysis resulting from bee sting (Fighera et al. 2007). Horses affected with piroplasmosis, neonatal isoerythrolysis and red maple intoxication (Alward et al. 2006) may present dark red urine. Cola-colored urine is also seen animals with degenerative myopathy. In these diseases, the lysis of the skeletal muscle fiber allows the leakage of myoglobin into the blood with consequent myoglobinemia. The size of the myoglobin molecule will enable it to pass through the renal glomeruli and will be eliminated in the urine, giving it the dark aspect. Cola-colored urine related to degenerative myopathy occurs in diseases such as exercise-associated myopathy of horses, capture myopathy in wild ruminants, poisoning by ionophore antibiotics in various species, poisoning by *Senna occidentalis* and *S. obtusifolia* in cattle and horses and nutritional myopathy (vitamin E deficiency in selenium) in several species, but mainly young ruminants (Barros 2016b).

Sago spleen. Amyloidosis is the extracellular deposition of amyloid and it is associated with several diseases. Amyloid means starch-like, but is a misnomer for the substance that has a high content of protein. Grossly the amyloid appears as a white, somewhat firm substance (Jones & Hunt 1983). One predisposing factor for amyloid production is chronic antigenic stimulation. Accordingly, amyloidosis occurs in the spleen of about 60% of the horses used for the production of antitetanic, antivenom and anti-diphtheric serum (Coelho 2002). In the spleen, the deposits either form between the lymphoid follicles or along the sinuses. As the deposits enlarge the lymphoid follicles, they become grossly visible as small nodules of white firm material (Jones & Hunt 1983). As the white pulp of the spleen is only 4-5% of the volume of the organ (Rooney & Robertson 1996) these white nodules contrast with the more abundant red pulp imparting a suggestion of a spleen sprinkled with sago pellets and hence the appellation, sago spleen (Jones & Hunt 1983).

Sago (in Portuguese: "sagu") is a starch extracted from several palm trees (e.g. *Metroxylon sagu* and *Cycas rumphii*) and used as staple food in different places in the world. Currently in Western countries, the term is used to refer to small round pellets derived from the cassava root (false sago) from which a dessert is made admixing wine, water, sugar, clover, and cinnamon. When the sago is cooked, the tiny pellets become swollen and translucent mimicking the appearance of amyloid. Sago spleen is an uncommon finding in the necropsy floor. However, some pathologists by analogy refer to marked lymphoid hyperplasia of the white pulp (Fig.6B) as sago spleen.

Blackberry jam. Anthrax, caused by the gram-positive bacterium *Bacillus anthracis*, is highly pathogenic to herbivorous animals and human beings. The disease has an acute to a peracute course. In affected cattle, the most prominent necropsy finding is a marked enlarged spleen (Valli et al. 2016). The consistency of the spleen is soft, the capsule may rupture, and a pasty floccular splenic pulp extrudes. This pulp is then colloquially referred to as blackberry jam (in Portuguese: "geleia de amora"). Since necropsy of a dead animal from anthrax is not recommended and since anthrax is currently a rare disease in most countries nowadays, this descriptive food analogy is of relatively small value.

MICROSCOPIC FINDINGS

Oat cells

In veterinary pathology, necrotic leukocytes with streaming nuclei that occur in the lung of cattle with shipping fever and pigs with contagious pleuropneumonia are called oat cells (in Portuguese: "células em grão de aveia") (Caswell & Williams 2016). The leukotoxins of *Mannheimia haemolytica* (Maheswaran et al. 1992) and *Actinobacillus pleuropneumoniae* (Choi et al. 2001) are believed to cause necrosis of neutrophils and perhaps macrophages with resultant fusiform shape of the nuclei and the streaming of the chromatin, resembling oat grains (Fig.7A).

Fried egg

Normal and neoplastic mast cells have a centrally located, round nucleus surrounded by abundant cytoplasm that gives a "fried egg" (in Portuguese: "ovo frito") appearance to the cell (Fig.7B) (Kiupel 2017). A similar pattern is observed

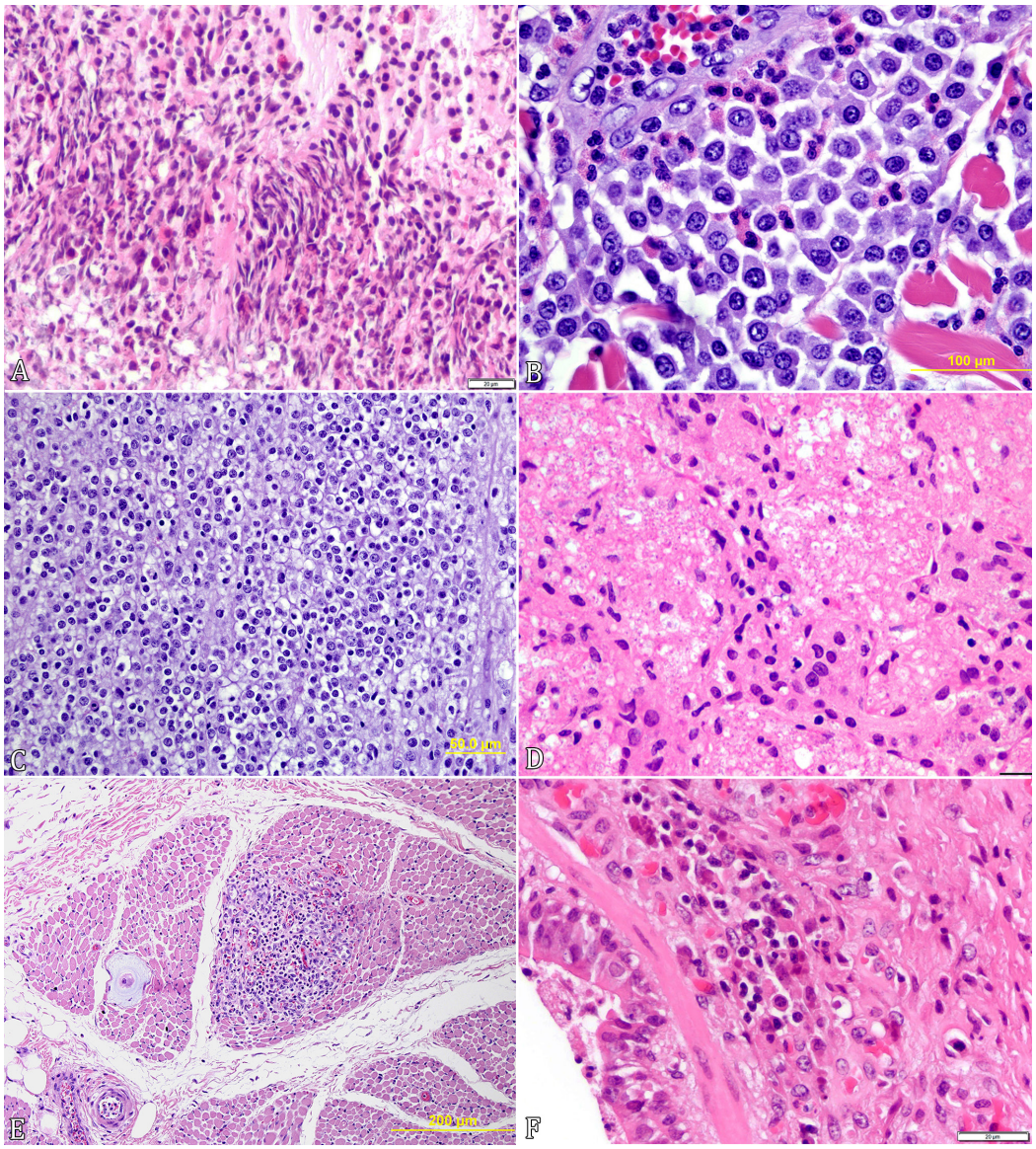


Fig.7. (**A**) Necrosuppurative bronchopneumonia, lung, pig; pulmonary actinobacillosis. Food analogy: oat cells. The alveolar lumina are obliterated by numerous elongated degenerated and necrotic leukocytes with nuclear streaming. HE, obj.40x. (**B**) Mast cell tumor, haired skin, dog. Food analogy: fried egg appearance. Well-differentiated mast cells containing abundant granular cytoplasm and round nucleus are intermixed with eosinophils. HE, obj.40x. (**C**) Oligodendroglioma, brain, dog. Food analogy: fried egg appearance. Neoplastic cells contain round, uniform, central nuclei surrounded by a halo of clear cytoplasm. The clear halo is an artifact of tissue fixation. HE, obj.20x. (**D**) Histiocytic interstitial pneumonia, lung, horse. Food analogy: honeycomb material. The alveolar spaces and septa are expanded by foamy, eosinophilic material, macrophages and numerous round, intra and extracellular, inconspicuous fungal bodies. HE, obj.60x. (**E**) Protozoal cyst, myocardium, dog. Food analogy: onion skin cyst. Encysted stages of *Hepatozoon americanum* contain a host cell (macrophage) in the center, surrounded by multilamellar, basophilic, amorphous material. HE, obj.20x. (**F**) Granulomatous bronchointerstitial pneumonia with lymphoid depletion and histiocytic intracytoplasmic botryoid inclusion bodies; lung; pig. Porcine circovirus-associated disease. Food analogy: botryoid inclusion bodies. HE, obj.40x.

in oligodendrogliomas. These are tumors derived from oligodendrocytes within the central nervous system and have a microscopic arrangement of cells with round, uniform, central nuclei surrounded by a halo of clear cytoplasm that is also comparable to the egg white of a fried egg (Fig.7C) (Reifenberger et al. 2000). The clear cytoplasm is actually an artifact of tissue processing from delayed fixation (Reifenberger et al. 2000) and is sometimes referred as honeycomb pattern (Vandevelde et al. 2012).

Honeycomb

Pneumocystis spp. are organisms classified as fungi that can cause disease in immunosuppressed animals and people (Skalski et al. 2015). Pneumonia is the most common clinical presentation. It is characterized histologically by foamy or "honeycomb" (in Portuguese: "favo de mel") material filling alveoli (Fig.7D) (Caswell & Williams 2016). This aspect is due to numerous intracellular and extracellular, round to crescent-shaped, clear fungal bodies that are about 8 µm in diameter (Caswell & Williams 2016). The organisms are inconspicuous on hematoxylin and eosin stain, but can be highlighted by methenamine silver stain (Caswell & Williams 2016).

Onion skin

Onion skin cysts are characteristic of American canine hepatozoonosis, caused by the protozoan *Hepatozoon americanum* (Cummings et al. 2005). The onion skin (in Portuguese: "pétalas de cebola") cyst (Fig.7E) is composed of multiple layers of acid mucopolysaccharide deposits that surround an enlarged macrophage that has been infected by a trophozoite (Cummings et al.2005). The cysts occur in several tissues, but are more common in striated muscle (Panciera et al. 1998).

Grape-like (botryoid) inclusions

The term "botryoid" is used to describe the viral inclusion bodies observed in infection by circoviruses, including porcine circovirus-2 and beak and feather disease virus in psittacine birds (Parrish 2010). From the Greek, botryoid meaning similar to a cluster of grapes (in Portuguese: "cacho de uva") (Stedman 2006) that resemble the deeply basophilic, round clusters of viral inclusion bodies that are observed in the cytoplasm of infected cells (Fig.7F) (Parrish 2010).

CONCLUSIONS

We emphasize here we do not recommend the use of macroscopic food analogies as descriptive terminology in the pathology reports. Their use should be applied in the academic setting to solidify technical learning in the long-term memory of the veterinary students since we believe adults must construct knowledge from knowledge they already possess, a principle long advocated by a distinguished Brazilian educator (Freire 2013).

On the other hand, veterinary pathologists will not hesitate to use terms like oat cells, onion skin cysts, and botryoid inclusion bodies when describing microscopic aspects of lesions or etiologic agents. Many of the analogies mentioned in this article were spontaneously born in the North hemisphere, yet most of them are well-known by Brazilian veterinary professionals. At any event, in this article, we consistently presented, within parenthesis, a translation into Portuguese for each food analogy.

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Original Article
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Cattle diseases in Mato Grosso do Sul, Brazil: a 24-year survey (1995-2018)[1]

Rayane C. Pupin[2]*, Paula V. Leal[3], Juliana P.L. Paula[4], Carolina C. Guizelini[4], Tessie B.M. Möck[2], Ricardo Antônio A. Lemos[2] and Danilo C. Gomes[2]

ABSTRACT.- Pupin R.C., Leal P.V., Paula J.P.L., Guizelini C.C., Möck T.B.M., Lemos R.A.A., & Gomes D.C. 2019. **Cattle diseases in Mato Grosso do Sul, Brazil: a 24-year survey (1995-2018).** *Pesquisa Veterinária Brasileira 39(9):686-695.* Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Avenida Senador Felinto Muller 2443, Vila Ipiranga, Campo Grande, MS 79074-460, Brazil. E-mail: rayane.pupin@gmail.com

We reviewed the records of 5,083 cattle necropsies performed from January 1995 to December 2018 and filed at the Laboratory of Anatomic Pathology (LAP) of the "Universidade Federal de Mato Grosso do Sul" (UFMS). These necropsies were performed either by LAP-UFMS faculty (22.33%) or by field veterinary practitioners (77.67%) who subsequently submitted material for histological evaluation at the LAP-UFMS. Conclusive diagnoses were reached in 46.21% of the protocols (2,349 cases), and approximately 65% of the cases were classified as inflammatory or parasitic diseases, with rabies being the most diagnosed disease (20.82% of total conclusive diagnosis). There were a large number of protocols in which the diagnosis was of nonsuppurative meningoencephalitis of unknown cause (NSMUC). Those were the main differentials for rabies and bovine herpesvirus-5 necrotizing meningoencephalitis (NME); that is, the number of rabies cases may be even higher if one considers that many cases of NSMUC might be undiagnosed rabies cases. Toxic and toxic-infectious diseases were the second most prevalent category, and botulism cases represented 41% of this category. The other categories corresponded to less than 20% of the total diagnoses and were distributed in decreasing order of frequency as degenerative diseases (9.79%), diseases caused by physical agents (3.87%), other diseases (2.13%), neoplasms and tumor-like lesions (1.79%), metabolic or nutritional disorders (1.75%) and congenital malformations (0.64%). The large number of inconclusive diagnoses was mainly due to improper conditions of mailed-in material for histopathological evaluation, namely, nonrepresentative samples of all organs, autolysis, and the absence of epidemiological and clinical-pathological information.

INDEX TERMS: Cattle diseases, Mato Grosso do Sul, Brazil, survey, pathology, epidemiology, livestock, causes of death.

RESUMO.- [**Doenças de bovinos em Mato Grosso do Sul, Brasil: uma pesquisa de 24 anos (1995-2018).**] Em um levantamento sobre doenças de bovinos, revisamos os protocolos de 5.083 necropsias de bovinos realizadas no período de janeiro de 1995 a dezembro de 2018 e arquivados no Laboratório de Anatomia Patológica (LAP) da Universidade Federal de Mato Grosso do Sul (UFMS). Essas necropsias foram realizadas pelos veterinários e professores do LAP-UFMS (22,33%) ou por médicos veterinários de campo (77,67%) que, posteriormente, submeteram ao LAP-UFMS material para avaliação histológica. Diagnósticos foram conclusivos em 46,21% dos casos protocolados (2349), e aproximadamente 65% deles foram classificados como doenças inflamatórias ou parasitárias, sendo a raiva a doença mais diagnosticada (20,82% dos diagnósticos conclusivos). Havia um grande número de protocolos nos quais o diagnóstico era de encefalite/meningoencefalite não supurativa de causa indeterminada, para o qual os dois principais diferenciais são raiva e meningoencefalite necrosante por herpesvírus bovino; isso sugere que o número

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[2] Laboratório de Anatomia Patológica (LAP), Faculdade de Medicina Veterinária e Zootecnia (FAMEZ), Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Felinto Muller 2443, Bairro Ipiranga, Campo Grande, MS 79074-460, Brazil. *Corresponding author: rayane.pupin@gmail.com

[3] Universidade Federal da Bahia (UFBA), Rua Adhemar de Barros 500, Ondina, Salvador, BA 40170-970, Brazil.

[4] Graduate Studies Program in Veterinary Science, FAMEZ-UFMS, Av. Senador Felinto Muller 2443, Bairro Ipiranga, Campo Grande, MS 79074-460.

de casos de raiva pode ser ainda maior, se considerarmos que muitos destes podem ser casos de raiva não diagnosticados adequadamente. As doenças tóxicas e toxi-infecciosas foram a segunda categoria mais prevalente; dentre elas, os casos de botulismo compuseram 41%. As demais categorias corresponderam a menos de 20% do total de diagnósticos e foram distribuídas em ordem decrescente de frequência, em doenças degenerativas (9,79%), doenças causadas por agentes físicos (3,87%), outras doenças (2,13%), neoplasmas e lesões tumoriformes (1,79%), distúrbios metabólicos ou nutricionais (1,75%) e malformações congênitas (0,64%). O grande número de diagnósticos inconclusivos deveu-se principalmente às condições inadequadas do material enviado ao LAP-FAMEZ para avaliação histopatológica, ou seja, amostras não representativas de todos os órgãos, autolisadas ou acompanhadas de poucas de informações epidemiológicas e clínico-patológicas.

TERMOS DE INDEXAÇÃO: Doenças de bovinos, Mato Grosso do Sul, Brasil, estudo retrospectivo, bovinos, levantamento, patologia, epidemiologia, causas de morte.

INTRODUCTION

The meat industry is an essential segment of the Brazilian economy, and beef is one of the main export products in the country. Any factor that may restrict trade in livestock products will result in a critical economic loss (Schulz et al. 2018). To maintain agribusiness, it is essential to maintain the health status of cattle herds. Thus, understanding which diseases affect cattle and their presentation and prevalence is paramount (Lucena et al. 2010).

Knowledge about the prevalence of different diseases that affect the cattle herds in a region is fundamental; with this knowledge, field practitioners and veterinarians can select from a list of differential diagnoses when reading laboratory or anatomopathological reports (Lucena et al. 2010).

Although there are few variations in diseases within the areas covered by each diagnostic laboratory (Rissi et al. 2010), epidemiological characteristics can vary widely between regions. There are also differences in the causes of mortality between regions due to different breeding systems (Mello et al. 2017, Rondelli et al. 2017, Santos et al. 2018).

Although some studies have investigated bovine diseases diagnosed in Mato Grosso do Sul (MS) (Salvador et al. 1998, Nakazato et al. 2000, Paiva et al. 2000, Brum et al. 2002, Lemos et al. 2005, Carvalho et al. 2006, Ferreira et al. 2008, Santos et al. 2012, Carvalho et al. 2013, Pinto et al. 2013, Ribas et al. 2013, Bacha et al. 2014, Carvalho et al. 2014, Souza et al. 2015, Faccin et al. 2016, Heckler et al. 2018, Guizelini et al. 2019, Pupin et al. 2019), there are no comprehensive data on the primary diseases that affect cattle in this region. We aimed to determine the frequency and epidemiological aspects of the primary bovine diseases diagnosed in MS by undertaking a retrospective study of 24 years (1995-2018).

MATERIALS AND METHODS

The records of cattle necropsies evaluated at the Laboratory of Anatomic Pathology (LAP) of the "Faculdade de Medicina Veterinária e Zootecnia" (FAMEZ) at the "Universidade Federal de Mato Grosso do Sul" (UFMS) from January 1995 to December 2018 were reviewed.

The exclusion criteria for the survey were as follows: (i) records on material from healthy cattle that were referred to the LAP-FAMEZ by the Ministry of Agriculture, Livestock and Supply from 2001 to 2007 during the Bovine Spongiform Encephalopathy surveillance program, (ii) material from experiments, (iii) material from states other than MS, (iv) samples with a diagnosis not associated with the animal's death, and (v) samples collected from a slaughterhouse.

Diagnoses were considered inconclusive when the case lacked gross and/or microscopic features that could explain the clinical signs and death or lacked characteristic clinical signs that could confirm the diagnosis even in the absence of anatomical lesions, as is the case of botulism and tetanus. The instances with conclusive diagnoses were grouped as previously described (Rondelli et al. 2017): inflammatory and parasitic diseases; toxic and toxic-infectious diseases; diseases caused by physical agents, metabolic or nutritional disorders, degenerative processes, congenital malformations, neoplasms and tumor-like lesions; and miscellaneous.

Botulism was diagnosed based on clinical signs, epidemiological findings and the absence of macroscopic and microscopic lesions in cases in which the necropsy was performed by the laboratory staff. Additionally, the detection of botulin toxin in the animal or in another animal from the same outbreak was considered a diagnostic criterion. The most frequently diagnosed diseases were individually discussed.

RESULTS

From January 1995 to December 2018, 5,083 bovine samples from necropsies performed by veterinarians of the LAP-FAMEZ staff or by field veterinary practitioners were evaluated; 1,135 (22.33%) were performed by LAP-UFMS faculty and 3,948 (77.67%) by field veterinary practitioners who subsequently submitted material to the LAP-UFMS for histological evaluation. Out of 2,734 necropsies (53,79%) with an inconclusive diagnosis, 2,459 (89.94%) were performed by field veterinary practitioners and 275 (11.06%) by LAP-FAMEZ faculty. Conclusive diagnoses were obtained in 46.21% of the cases (2,349 necropsies). The stratification of the disease categories is shown in Figure 1.

The main cause of deaths was inflammatory and parasitic diseases representing more than 65% of the conclusive diagnoses (Table 1). The group with the second highest number of diagnoses was toxic diseases and toxic-infectious diseases, corresponding to 356 diagnoses (Table 2). Mortalities caused by physical agents are shown in Table 3. Those produced by metabolic or nutritional disorders are shown in Table 4, and congenital malformations in Table 5. There were 42 cases of neoplasms and tumor-like lesions found at necropsy (Table 6). Diseases that could not be included in any of the categories were classified as "miscellaneous" and are listed in Table 7.

DISCUSSION

In this 24-year study, we observed that over time, the number of necropsies performed gradually decreased, as has also been observed in similar surveys (Oliveira et al. 2012), although the cause of this decrease is unknown. This decrease suggests that although necropsy is often the only method of diagnosis, it is often neglected (Peixoto & Barros 1998). Usually, financial concerns prevent producers from seeking help. The request for necropsy is most frequent when (i) death occurs in cattle of high commercial value; (ii) the

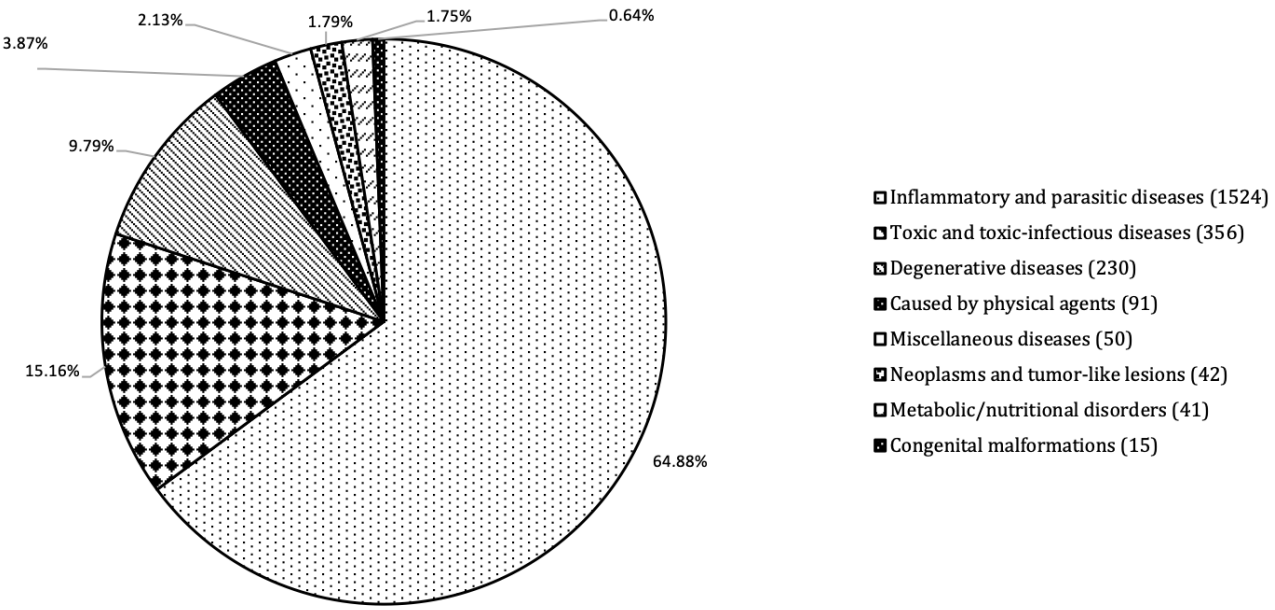


Fig.1. Necropsies performed in cattle from 1995 to 2018 in Mato Grosso do Sul, and number of cases of each category.

Table 1. Inflammatory and parasitic diseases (n=1,524) in cattle in Mato Grosso do Sul (January 1995-December 2018)

Disease	N[a]	%	Disease	N[a]	%
Rabies	489	32.09	Myocarditis (unknown cause)	3	0.2
Non-suppurative meningoencephalitis (unknown cause)	319	20.93	Endocarditis	3	0.2
Necrotizing herpesviral meningoencephalitis	147	9.65	Parasitic enteritis	3	0.2
Septicemia	85	5.58	Leptospirosis	3	0.2
Tick fever	84	5.51	Pericarditis	3	0.2
Blackleg	57	3.74	Pleuritis + pericarditis	3	0.2
Bacterial pneumonia	54	3.54	Verminous pneumonia	3	0.2
Other pneumonia	31	2.03	Anemia and hypoproteinemia (*Rhipicephalus microplus*)	2	0.13
Peritonitis	24	1.57	Bovine viral diarrhea	2	0.13
Osteomyelitis	21	1.38	Mastitis	2	0.13
Cerebral babesiosis	21	1.38	Mycotic pneumonia	2	0.13
Salmonelosis	18	1.18	Listeriosis (presumptive)	2	0.13
Malignant catarral fever	16	1.02	Diphteric abomasitis	1	0.07
Enteritis (unknown cause)	16	1.02	Cervical abscesses	1	0.07
Brain abscesses	14	0.92	Tracheal compression by abscess	1	0.07
Hemonchosis	12	0.79	Malignant edema	1	0.07
Bacterial meningoencephalitis	10	0.66	Generalized mycotic infection	1	0.07
Enteric colibacillosis	9	0.59	Meningoencephalitis (*Histophilus somni*)	1	0.07
Cystitis	8	0.52	Meningoencephalitis with mixed inflammatory infiltrate	1	0.07
Hepatic abscesses	7	0.46	Septic necrotizing myositis	1	0.07
Eimeriosis	5	0.33	Septic osteomalacia	1	0.07
Estomatitis/esophagitis (unknown cause)	5	0.33	Suppurative otitis	1	0.07
Pituitary abscess	5	0.33	Parasitism (*Trichostrongylus* sp.)	1	0.07
Tuberculosis	5	0.33	Pyelonephritis and urethritis	1	0.07
Dermatophilosis	4	0.26	Pneumonia BRSV[b]	1	0.07
Eosinophilic meningoencephalitis	4	0.26	Bovine infectious rhinotracheitis (presumptive)	1	0.07
Rhinitis (unknown cause)	4	0.26	Trypanosomiasis (presumptive)	1	0.07
Anaplasmosis + salmonelosis	4	0.26			

[a] Number, [b] bovine respiratory syncitial virus.

number of fatalities is abnormally high; (iii) there are livestock insurance investigations; or (iv) there is a judicial dispute (Waldner et al. 2009). From the perspective of farmers, it is easier and cheaper to send fragments of viscera and/or samples for clinical analysis than to transport a cadaver to the lab (Watson et al. 2008). Transportation from the property to the laboratory is a challenge, as it frequently requires travel over long distances. Surveys from Sweden (Svensson et al. 2006) and the United Kingdom (Watson et al. 2008) conclude

Table 2. Toxic and toxic-infectious diseases (356) in cattle diagnosed in LAP-UFMS (January 1995-December 2018)

Disease	Number of cases	%
Botulism	146	46.01
Poisoning by *Vernonia* sp.	53	14.89
Poisoning by *Senna* sp.	28	7.87
Toxic hepatic fibrosis	23	6.46
Poisoning by *Brachiaria* sp.	19	5.34
Tetanus	15	4.21
Poisoning by *Amorimia* sp.	11	3.09
Toxic nephrosis associated with crystals (unknown cause)	10	2.81
Toxic nephrosis (unknown cause)	8	2.25
Photosensitization (unknown cause)	6	1.69
Poisoning by *Enterolobium contortisiliquum*	6	1.69
Poisoning by citrus pulp	5	1.4
Poisoning by salt (NaCl)	4	1.12
Lead poisoning	4	1.12
Poisoning by *Stryphnodendron fissuratum*	4	1.12
Urea poisoning	3	0.84
Poisoning by abactin	3	0.84
Poisoning by amitraz	2	0.56
Poisoning by cassava (*Manihot esculenta*)	2	0.56
Poisoning by *Solanum malacoxylon*	2	0.56
Poisoning by *Crotalaria* sp.	1	0.28
Poisoning by terramycin	1	0.28

Table 3. Diseases caused by physical agents (n=91) in cattle in Mato Grosso do Sul (January 1995- December 2018)

Disease	Number of cases	%
Hypothermia	35	35.4
Fracture	14	14.1
Trauma	14	14.1
Aspiration pneumonia	14	14.1
Hardware disease	3	3.03
Water deprivation	2	2.02
Diphteric rumenitis due to ingestion of caustic chemical	2	2.02
Abomasal obstruction	2	2.02
Spinal cord compression by bone overgrowth	1	1.01
Milk-induced necrotic rumenitis	1	1.01
Traumatic rumenitis	1	1.01
Sand impaction	1	1.01
Electrocution (presumptive)	1	1.01

Table 4. Metabolic and nutritional disorders in cattle in Mato Grosso do Sul (January 1995-December 2018); percent over 41 cases (n=41)

Disease	Number of cases	%
Ruminal acidosis	10	24.39
Cobalt deficiency (presumptive)	7	17.07
Starvation	6	14.63
Ketosis	6	14.63
Hypocalcemia post-partum	3	7.32
Vagal indigestion	3	7.32
Bloat	3	7.32
Osteomalacia	1	2.44
Vitamine E and selenium deficiency	1	2.44
Urethral obstruction (urolithiasis)	1	2.44

Table 5. Congenital malformations (n=15) in cattle in Mato Grosso do Sul (January 1995-December 2018)

Disease	Number of cases	%
Arthrogryposis	3	20
Patent urachus	2	13.33
Multiple malformations	2	13.33
Anal atresia	1	6.67
Multiple cardiac defects	1	6.67
Umbilical hernia	1	6.67
Vaginal malformations	1	6.67
Forelimbs malformations	1	6.67
Palatoschisis (cleft palate)	1	6.67
Patent ductus arteriosus	1	6.67
Persistent right aortic arch	1	6.67

Table 6. Neoplasms and tumor-like lesions (n=42) in cattle in Mato Grosso do Sul (January 1995-December 2018)

Type of lesion	Number of cases	%
Bovine enzootic leukosis	9	21.43
Lymphoma	6	14.29
Squamous cell carcinoma	6	14.29
Hepatocellular carcinoma	3	7.14
Disseminated anaplastic carcinoma	2	4.76
Renal carcinoma	2	4.76
Cholangiocellular carcinoma	2	4.76
Fibrosarcoma	1	2.38
Mesothelioma	1	2.38
Undifferentiated pulmonary carcinoma	1	2.38
Esophagic fibromas	1	2.38
Liposarcoma	1	2.38
Meningioma	1	2.38
Metastatic pheocromocytoma (lung)	1	2.38
Metastatic seminoma (disseminated)	1	2.38
Olfactory neuroblastoma (presumptive)	1	2.38
Choroid plexus papilloma	1	2.38
Embryomal rhabdomyosarcoma	1	2.38
Undifferentiated sarcoma	1	2.38

Table 7. Miscellaneous diseases (n=50) in cattle diagnosed at LAP-UFMS (January 1995-December 2018)

Disease	Number of cases	%
Acute hepatic necrosis (unknown cause)	14	28
Heart failure	10	20
Muscle rupture	4	8
Hypovolemic shock	4	8
Abomasal ulcer	2	4
Snake bite	2	4
Amiloidosis	1	2
Omasal impactation	1	2
Evisceration and intestinal perforation	1	2
Pulmonary hemorrhage	1	2
Hypomyelinogenesis	1	2
Chronic renal failure	1	2
Intussusception	1	2
Abomasal glandular hyperplasia and metaplasia	1	2
Necrosis of cerebral white matter (unknown cause)	1	2
Megaesophagus	1	2
Senility	1	2
Vena cava symdrome	1	2
Abomasal torsion	1	2
Intestinal torsion	1	2

that this is the leading reason reported by producers for not sending animals to necropsy at the laboratory.

The percentage of inconclusive cases was higher than that (14.2-37.1%) in similar surveys from Rio Grande do Sul, southern Brazil (Lucena et al. 2010, Schild et al. 2013, Mello et al. 2017), but similar to that observed in the neighboring state of Mato Grosso (Rondelli et al. 2017). In the current study, almost 90% of the necropsies were performed by field veterinary practitioners, and tissue samples were sent to LAP-FAMEZ. These samples were often suboptimal for histopathological evaluation because (i) there were no representative samples of all organs associated with the disease, (ii) tissues were autolyzed, and (iii) there was lack of epidemiological and clinical-pathological information. This might explain the relatively high number of inconclusive diagnoses in mailed-in cases compared with cases worked up by the LAP-FAMEZ staff. Similar discrepancies have been reported by others (Lemos et al. 2005, Lucena et al. 2010, Rissi et al. 2010, Ribas et al. 2013, Rondelli et al. 2017).

Similarly to what is described in Mato Grosso (Rondelli et al. 2017), in our study, the five most common diagnosis (rabies, NSMUC, polioencephalomalacia, meningoencephalitis caused by BoHV and botulism) are characterized by neurological signs. This aspect can suggest some hypothesis: (i) because of the great importance of rabies in MS, when people see a bovine with neurological signs, they normally suspects of rabies and tend to send the animal to necropsy or do the procedure to collect samples to send to a diagnostic laboratory; (ii) normally, these animals have a clinical course of some days, so it's possible to see the evolution of the disease and decide about do or not the necropsy or (iii) these diseases are really the main cause of cattle death in the state.

Inflammatory and parasitic diseases were the leading causes of death of cattle in the present study, similar to results reported by other surveys all over the country (Lucena et al. 2010, Assis-Brasil et al. 2013, Mello et al. 2017, Rondelli et al. 2017).

Rabies was the most frequently diagnosed disease. This confirms data from other states in Brazil that indicate rabies as a critical problem nationwide (Galiza et al. 2010, Rondelli et al. 2017, Terra et al. 2018). Rabies corresponded to 9.62% of cattle diseases in the current study; this value is 20.82% when only conclusive diagnoses are considered and represents 32.09% of all inflammatory and parasitic infections. This number is much higher than that described in studies conducted in southern Brazil, where rabies corresponds to 4.67% (Lucena et al. 2010) and 3.67% (Schild et al. 2013) of cases with a conclusive diagnosis. Rabies morbidity rates were 0.008%-64%. The number of rabies diagnoses is potentially even higher in the present study since the second most frequent diagnosis (n=319) of inflammatory and parasitic diseases is nonsuppurative meningoencephalitis of unknown cause (NSMUC), which could be cryptic cases of rabies. Another differential would be necrotizing meningoencephalitis (NME) by bovine herpesvirus (BoHV) (Rondelli et al. 2017). However, rabies lacks the severe cerebrocortical necrosis found in BoHV-induced disease (Rissi et al. 2007, 2010).

Among the diagnoses of NSMUC, 295 (92.48%) were made on mailed-in bovine tissues. This negatively influences the number of confirmed diagnoses because fragments from the brain, cerebellum and spinal cord were sent in only 37 cases of NSMUC. The nonreferral of the entire central nervous system (CNS), mainly portions of the spinal cord, for evaluation reduces the chances of diagnosing rabies, as the lesion distribution and amount of antigen vary between CNS sites but are consistently present in the spinal cord (Bingham & Van der Merwe 2002, Bassuino et al. 2016). The submission of fragments from spinal cord, cerebellum, and brainstem, especially from livestock that have been euthanized, is critical for diagnosis since there is a high risk of false negatives when only the cerebral hemispheres are evaluated (Lemos 2005). In addition, sending the GRH complex (trigeminal ganglion, *rete mirabile* and hypophysis) improves diagnosis accuracy since more than 78% of rabid cattle have lymphoplasmacytic ganglioneuritis with or without inclusion bodies (Lemos et al. 2005, Rech et al. 2006, Marcolongo-Pereira et al. 2011). Accordingly, the number of diagnoses of NSMUC decreased beginning in 2004, probably in association with the publication of the manual on procedures for the diagnosis of bovine CNS diseases (Barros & Figueiredo 2003) by the Ministry of Agriculture, Livestock and Supply.

A previous survey involving CNS diseases of cattle in MS (Ribas et al. 2013) suggested the existence of cyclicity in the occurrence of rabies. However, in the current study, there was a gradual increase in the number of diagnoses between 1995 and 2002, which was paralleled by an increase in the number of bovine samples evaluated. This was followed by a decrease, with small oscillations in subsequent years, until 2018 (Fig.2). Similar to what was described in a survey in Paraná, southern Brazil (Dognani et al. 2016), the occurrence of rabies in our study varied from year to year, but the existence of cyclicity was questionable and in that study (Dognani et al. 2016) was considered to be 18 years, however, the authors

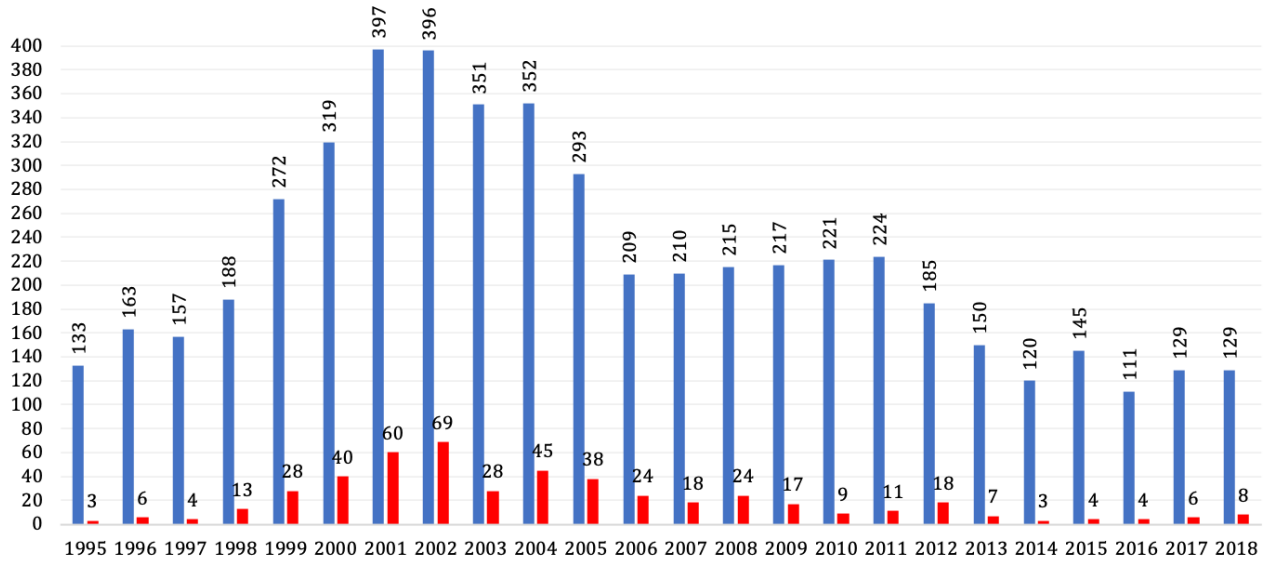


Fig.2. Total number of necropsies in cattle (blue bar) and number of cases of bovine rabies (red bar) in Mato Grosso do Sul from 1995 to 2008.

did not back up this assertion of cyclicity with hard data. In our study, it was not possible to verify cyclicity in rabies occurrence because the number of cases diagnosed does not reflect its actual occurrence due to underreporting of cases and the fact that we considered only the materials referred for histopathological evaluation. The number of cattle deaths from rabies reported in our study is smaller than that determined by other research performed at our laboratory because unlike in previous research (Mello et al. 2019), our data do not include all cases of cattle rabies worked up by the official sanitary defense laboratory from MS.

The age of rabid cattle in the current survey varied from 30 days to 16 years. The highest number of cases (35.99%) occurred in calves aged up to one year, followed by cattle aged 13 to 24 months (26.17%). A similar age distribution was reported from the state of Paraíba (Lima et al. 2005). These authors attributed the highest occurrence of cases in young cattle to the weak immunity of this age group, which may be due to lack of booster doses at 30-40 days after an earlier (prime) dose or to a delay in prime vaccination, which should be performed between the third and fourth months of life.

NME by BoHV (147 cases) was the second most common inflammatory disease. More than 70% of individuals (104 cases) affected by NME were 12- to 24-month-old cattle. NME by BoHV-5 is typically described in the literature as a disease of young calves (Colodel et al. 2002, Elias et al. 2004, Halfen & Riet-Correa 2007, Galiza et al. 2010, Cagnini et al. 2017, Middleton 2017, Blume et al. 2018); however, some reports indicate that the disease can occur in adult cattle (Roels et al. 2000, Rissi & Barros 2013). Morbidity was up to 11%. Stress factors that could have induced viral reactivation (Halfen & Riet-Correa 2007), such as weaning, castration, vaccination, deworming, pasture change, feed change and transportation (Rissi et al. 2010, Santos et al. 2018), were not mentioned in most protocols. In cases where this information was available, the main stress factor mentioned was transportation and mixing of large numbers of calves (13 cases). Finally, one should consider that at least some cases of NME by BoHV were associated with recent viral infection and not with reactivation of previous infection (Lemos 2005).

Cases of meningoencephalitis caused by BoHV have often been attributed to BoHV-5 (Rissi et al. 2007) because this is the type most commonly associated with neurological diseases. However, meningoencephalitis caused by BoHV-1 was already described (Roels et al. 2000, Rissi et al. 2008) and cases were also experimentally reproduced by this herpesvirus type (Marin et al. 2015). So, as in our study we didn't do the characterization of the agent in the cases diagnosed, it was considered just herpetic meningoencephalitis.

The typical gross brain lesions for NME by BoHV described in the literature as flattening and yellow discoloration of the cerebral gyri and softening of the cerebral cortex (Elias et al. 2004, Rissi et al. 2010, Rissi & Barros 2013, Blume et al. 2018) were reported in only 41% of our cases. Again, we interpreted this as due to unreported gross findings, since no gross description of the brain was provided in 50 cases. Very acute lesions may be easily neglected when necropsy is performed by a nonpathologist veterinarian (Rissi et al. 2007); furthermore, since the brain is softened, collection artifacts are prone to occur and can be interpreted as nonlesions.

There were 85 cases (3.62% of conclusive cases) of septicemia. Septicemia was most frequently diagnosed in young calves. Seventy-nine (93%) of the individuals in the septicemic category were calves less than 12 months old. Forty-four were calves up to 30 days old, 28 were calves between 31 and 180 days old, and five were calves between six and 12 months old. Only five adult cattle were affected; in two individuals, it was possible to identify the source of the primary infection: one dairy cow had severe mastitis, and the Nellore heifer developed postpartum metritis and subsequent septicemia.

Results of bacteriological culture were present in 12 of the 85 reports. The following bacteria were identified: *Escherichia coli* (in 5 cases), *Pseudomonas* sp. (3 cases), *Streptococcus* sp. (1 case), *Corynebacterium* sp. (1 case), *Acinetobacter* sp. (1 case) and *Trueperella pyogenes* (1 case). Despite the low number of tests performed, the results are similar to those described in the literature, which mention gram-negative bacteria, especially *E. coli*, as the most common pathogens associated with bacterial septicemia in calves (Fecteau & George 2004,

Dore & Smith 2017). When outbreaks of sepsis and meningitis are diagnosed on a farm, risk factors or management issues must be investigated. The most common are contaminated colostrum, improper navel asepsis, overcrowding, and poor hygiene (Dore & Smith 2017).

Tick fever was the seventh most frequent disease (3.58% of bovine deaths). This number is lower than that observed southern Rio Grande do Sul, southern Brazil (Almeida et al. 2006), which is expected since midwestern Brazil is characterized by enzootic stability and southern Brazil by enzootic instability (Farias 2007). The animals ranged in age from two days to 11 years old, and most were zebuine cattle, probably because of the high number of zebuine cattle raised in MS due to the lack of a susceptibility difference between zebuine and taurine cattle (Jonsson et al. 2008). Epidemiological, clinical and pathological aspects of bovine babesiosis diagnosed in MS have already been published (Pupin et al. 2019).

In the group of toxic and toxic-infectious diseases, 146 (41.01%) were cases of botulism. The diagnoses were made in a similar way as in other states in Brazil (Galiza et al. 2010, Schild et al. 2013, Rondelli et al. 2017, Terra et al. 2018). The other diagnostic criterion - detection of botulin toxin - was not described in previous surveys (Galiza et al. 2010, Schild et al. 2013, Rondelli et al. 2017, Terra et al. 2018) but was used in one recent outbreak in MS (Guizelini et al. 2019). Similarly, in MT (Rondelli et al. 2017), botulism was the main toxic disease diagnosed; the opposite was observed in Rio Grande do Sul, southern Brazil, where poisoning by plants was the main toxic cause of bovine death (Lucena et al. 2010, Schild et al. 2013, Santos et al. 2019). Morbidity ranged from 0.08 to 64.7%.

The cases occurred in small or large outbreaks affecting from 14-1,100 cattle. We followed the course of 11 outbreaks. Samples of 50 cattle were analyzed. Two of those outbreaks occurred in feedlots - one associated with the feeding of moldy hay (Soares et al. 2018a) and the other of contaminated rehydrated corn (Guizelini et al. 2019). The other nine were observed in extensively raised cattle at pasture. Seven of those nine were associated with osteophagia, and two with drinking from a contaminated pond. The number of cattle affected in the other outbreaks was 2-20. Because samples from only one bovine per occurrence were evaluated, these cases were not listed as outbreaks. Most of the cases occurred in cattle raised at pasture in farms where carrion was left in the pastures (Lemos 2005, Rondelli et al. 2017). This reinforces the notion that most cases of botulism in cattle at pasture are related to osteophagia, in contrast to observations in northeastern Brazil (Galiza et al. 2010), where outbreaks occur primarily in cattle fed chicken litter.

Poisoning by *Vernonia* sp. was the main plant poisoning diagnosed and represented 2.26% of all diagnoses and was the main toxic disease associated with plant poisoning. Of these cases, 29 were already published (Brum et al. 2002). The remaining cases occurred in cattle over one year old and in seven farms in the form of large outbreaks affecting 17-200 cattle. Epidemiological data and information on the economic impact caused by this type of plant poisoning are available (Soares et al. 2018b).

Degenerative diseases were the third most frequent group. In this category, only two disorders were diagnosed: polioencephalomalacia (PEM) (205 cases) and muscular necrosis of unknown cause (25 cases).

PEM was the central degenerative disease, as is typically described in other regions of Brazil (Lucena et al. 2010, Rondelli et al. 2017). It represented more than 89% of the cases in this disease category and was the third most prevalent diagnosis of all cattle diseases, corresponding to 8.73% of the conclusive diagnoses, a number well above that described in southern Rio Grande do Sul (Lucena et al. 2010, Schild et al. 2013). This suggests that some environmental aspects present in Midwestern Brazil might favor the development of the disease. Morbidity was generally low (0.016-23%). PEM is reported as a common problem in feedlot calves between six and 18 months of age in the USA (Dore & Smith 2017). In the present study, the majority of PEM cases involved cattle at pasture aged between 13 and 36 months (62%). The etiology was not determined in any case of PEM, as is usually the case (Lemos 2005, Rissi et al. 2010, Rondelli et al. 2017, Terra et al. 2018). However, approximately 70% of the necropsy records did not contain any epidemiological information, which definitively and profoundly compromised the identification of possible agents involved.

Death of cattle due to hypothermia (35 cases) corresponded to the most frequent disease in the category of diseases caused by a physical agent, accounting for 38.46% of cases. The vast majority of instances (33 cases) occurred in the period of 2000-2010 and has been previously described (Santos et al. 2012). There were an additional two outbreaks of this condition on a farm in which, of a total of 500 cattle, 40 became ill, and 30 died. A sudden drop in temperature the day before the deaths and the clinical signs and pathological findings were similar to those in previously published cases (Santos et al. 2012).

The number of neoplasms and tumor-like lesions is much lower than that described in other Brazilian surveys (Lucena et al. 2010, 2011, Reis et al. 2017). We attribute this discrepancy to a large number of cases of chronic bracken fern (*Pteridium aquilinum*) intoxication in cattle in the south, which is known to induce various types of tumors in the urinary bladder and squamous cell carcinoma in the upper alimentary tract (Lucena et al. 2010), and to the high incidence of lymphomas associated with infection with the retrovirus of bovine enzootic leukosis (Reis et al. 2017). In addition, more than 90% of the samples evaluated in those surveys (Lucena et al. 2010, 2011) were from dairy cattle that remained in the herd longer (reaching the "tumor age") than did beef cattle. In another survey in southern Brazil involving only dairy cows (Mello et al. 2017), neoplasia was the second most frequent cause of death, with lymphoma as the leading neoplasm.

The remaining category had a low number of cases. We believe that the economic losses resulting from these cases were not significant since these cases are infrequent in cattle (Lucena et al. 2010, Rondelli et al. 2017). Nutritional and metabolic disorders corresponded to a small proportion of the diagnoses possibly because most animals were beef cattle raised in an extensive system at pasture.

CONCLUSIONS

The main causes of bovine death in Mato Grosso do Sul, Central-Western Brazil, identified in 24 years were inflammatory and parasitic diseases, toxic diseases and degenerative diseases.

The main diagnose was rabies, followed by nonsuppurative meningoencephalitis of unknown cause, polioencephalomalacia,

necrotizing meningoencephalitis by bovine herpesvirus and botulism, all diseases that causes neurological signs.

We observed a decrease in the number of samples from bovine's necropsies evaluated over the years, but we couldn't suggest possible reasons for it.

Deaths caused by physical agents, metabolic and nutritional disorders and neoplasms and tumor-like lesions represented a low percentage of the cases. This is related to cattle production system in MS, which is predominantly in pastures and for beef purposes.

The large number of inconclusive diagnoses is associated with failure to collect data and samples, which reinforces the vulnerability of the diagnosis process.

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Necrohemorrhagic enteritis outbreak in a cattle feedlot in Nova Crixás, Goiás, Brazil[1]

Rafael C. Sanguanini[2], Luna S. Girão[2], Rodrigo Otávio S. Silva[3], Francisco Carlos F. Lobato[3], Regiani N.G. Porto[2], Cairo Henrique S. Oliveira[4] and Veridiana M.B.D. Moura[2]*

ABSTRACT.- Sanguanini R.C., Girão L.S., Silva R.O.S., Lobato F.C.F., Porto R.N.G., Oliveira C.H.S. & Moura V.M.B.D. 2019. **Necrohemorrhagic enteritis outbreak in a cattle feedlot in Nova Crixás, Goiás, Brazil.** *Pesquisa Veterinária Brasileira 39(9):696-699.* Setor de Patologia Animal, Escola de Veterinária e Zootecnia, Universidade Federal de Goiás, Avenida Esperança s/n, Campus Universitário Samambaia, Goiânia, GO 74690-900, Brazil. E-mail: vdmoura@hotmail.com

This study described an outbreak of necrohemorrhagic enteritis in a beef cattle feedlot in Nova Crixás, State of Goiás, Brazil, with emphasis on epidemiological, lesional, and laboratory aspects. Visits to the property were carried out and a necroscopic examination was performed on the bovine cadavers (N=57), which presented similar macroscopic alterations. Epidemiological data were collected, mainly referring to the feeding management of animals, and tissue samples were submitted to histopathological examination. Samples of feces and intestinal contents were also collected for bacterial isolation and PCR genotyping to detect the etiological agent, being confirmed *Clostridium perfringens* type A strains in 100% of the samples. Furthermore, 33.3% of strains isolated from intestinal contents and 40% of those isolated from feces were positive for beta-2 encoding gene. Considering the history, macroscopic and microscopic findings, as well as bacterial isolation and PCR, the diagnosis of bovine necrohemorrhagic enteritis was determined.

INDEX TERMS: Necrohemorrhagy, enteritis, outbreak, cattle, feedlot, Goiás, Brazil, *Clostridium perfringens*, alpha toxin, hemorrhagic bowel syndrome, bovine.

RESUMO.- [**Surto de enterite necro-hemorrágia em um confinamento de bovinos no município de Nova Crixás, Goiás, Brasil.**] Descreve-se um surto de enterite necro-hemorrágica em um confinamento de bovinos de corte no município de Nova Crixás, Estado de Goiás, Brasil, com ênfase nos aspectos epidemiológicos, lesionais e laboratoriais. Foram realizadas visitas à propriedade e todos os cadáveres bovinos (N=57) foram submetidos ao exame necroscópico, os quais apresentaram alterações macroscópicas semelhantes. Foram compilados dados epidemiológicos, sobretudo referentes ao manejo alimentar dos animais e amostras de tecido foram submetidas a exame histopatológico. Foram colhidas, também, amostras de fezes e conteúdo intestinal para isolamento bacteriano e genotipagem por PCR para detecção do agente etiológico, sendo confirmadas estirpes de *Clostridium perfringens* tipo A em 100% das amostras. Ainda, 33,3% das cepas de *Clostridium perfringens* isoladas no conteúdo intestinal e 40% daquelas isoladas nas fezes foram positivas para o gene codificador da toxina beta-2. Considerando o histórico, os achados macroscópicos e microscópicos, o isolamento bacteriano e o PCR, foi estabelecido o diagnóstico de enterite necro-hemorrágica por *C. perfringens* tipo A.

TERMOS DE INDEXAÇÃO: Surto, enterite, necro-hemorragia, confinamento, bovinos, Goiás, Brasil, alfa toxina, *Clostridium perfringens*, síndrome do intestino hemorrágico.

INTRODUCTION

Bovine necrohemorrhagic enteritis is a clostridiosis caused by *Clostridium perfringens* type A strains (Goossens et al. 2016a), which presents necrotic and hemorrhagic lesions in the small intestine and sudden death of apparently healthy

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[2] Setor de Patologia Animal, Departamento de Medicina Veterinária, Escola de Veterinária e Zootecnia, Universidade Federal de Goiás (UFG), Avenida Esperança s/n, Campus Universitário Samambaia, Goiânia, GO 74690-900, Brazil. *Corresponding author: vdmoura@hotmail.com

[3] Laboratório de Bacterioses e Pesquisa, Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Avenida Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil.

[4] Ministério da Agricultura, Pecuária e Abastecimento, Praça Cívica 100, 3º andar, Goiânia, GO 74003-010.

animals (Lebrun et al. 2010, Verherstraeten et al. 2013, Goossens et al. 2016a, 2016b, 2017). Although this disease presents a low morbidity and individual cases are observed sporadically, the high lethality, the rapid course to death, and the almost impracticable treatment confer economic importance to this disease in cases of outbreak, and prophylaxis should be prioritized (Lebrun et al. 2010, Goossens et al. 2016a, 2016b, 2017). This study described an outbreak of bovine necrohemorrhagic enteritis in beef cattle feedlot located in Nova Crixás, Goiás, Brazil. Epidemiological, *post mortem*, and laboratory aspects of this disease are presented.

MATERIALS AND METHODS

A total of 57 deaths of cattle were observed from January to March, 2017 in a cattle feedlot in the municipality of Nova Crixás, Goiás, Brazil. All animals, although apparently healthy and with a good body score, presented sudden death without premonitory clinical signs associated with marked abdominal distension and accelerated putrefaction process. Anatomopathological examinations were performed on the corpses of the 57 dead animals, which presented the same pattern of lesions, characterized especially by small intestine loops of blackish red coloration and markedly distended due to the accumulation of a large amount of gas. The opening of the loops of the small intestine also showed a large amount of bloody fluid content and a diffuse and markedly hemorrhagic mucosa, showing streaks of blackish coloration (Fig.1). In addition, irregularities were verified in feeding times of animals, changes in diet composition, and large intervals between one supply and another of the ration up to 48 hours before death episodes.

After necropsy performed on fresh corpse of animal found dead, fragments of small intestine fixed in 10% buffered formalin were sent for histopathological examination. Samples were processed according to routine techniques for paraffin embedding and histological sections were stained with hematoxylin and eosin (HE) and Gram techniques. In addition, three samples of intestinal contents and ten samples of feces, collected from three animals that died from March 19th to 25th, 2017, were submitted for isolation and genotyping of *Clostridium perfringens* as previously described (Silva et al. 2016).

RESULTS

The histomorphologic evaluation of the small intestine showed marked edema, extending from the superficial stratum to the deepest part of the intestinal wall. The mucosa layer presented diffuse necrosis of the villi in association with hemorrhagic content and a marked mononuclear and polymorphonuclear inflammatory infiltrate. Gram-positive bacillary structures of diffuse distribution, consistent with *Clostridium* spp., were also observed in the mucosa layer. Some crypts were preserved, but with degenerate cells. Diffuse and marked edema was observed in the submucosa, as well as dilation of blood and lymphatic vessels (Fig.2). The muscular layers showed interstitial edema, degeneration, and necrosis of the myenteric plexus. In addition, bacterial isolation and PCR genotyping confirmed *C. perfringens* type A strains in 100% of the samples of intestinal contents and feces, of which 33.3 and 40% respectively, were beta-2 positive. All the samples of intestinal contents had high bacterial counts (>10^6CFU/g). In contrast, 20% of feces samples had high bacterial counts and the remainder presented counts of 10^4CFU/g, which infers a microenvironment favorable to acute bacterial proliferation in the intestines of animals, resulting in the death of animals before high counts of bacteria could be seen in the feces. Based on the history, epidemiological pattern, histomorphologic findings, and microbiological results, the definitive diagnosis was necrohemorrhagic enteritis or hemorrhagic bowel syndrome.

DISCUSSION

Necrohemorrhagic enteritis in bovine is an important cause of sudden death (Lebrun et al. 2010, Verherstraeten et al. 2013, Goossens et al. 2016a). It occurs mainly in calves under an intensive breeding system, but this disease also affects adult animals. The main cause is a diet rich in proteins and carbohydrates, which, in excess, can overcome the digestive and absorption capacity of the digestive system of animals and, therefore, ends up serving as a substrate for the proliferation of *Clostridium perfringens* (Lebrun et al. 2010, Goossens et al. 2016a). During the period from January to March of 2017, there were five changes in the composition of the diet offered to the animals and the episodes of sudden death occurred up to 48 hours after its supply. However, abrupt changes in the regularity of feeding, delays in the ration distribution, and logistic intercurrences that involved large intervals without food available to the animals were related to episodes of sudden death due to necrohemorrhagic enteritis. Specifically, from March 18th to 19th, 2017, the animals remained 15 hours with the empty troughs, with 13 deaths on the two subsequent days. Stress due to sudden changes in diet composition and amount of food available is indicated as a predisposing factor to necrohemorrhagic enteritis, being observed from 24 to 36 hours before death (Lebrun et al. 2010, Verherstraeten et al. 2013, Goossens et al. 2016a). Transport of animals, regrouping, problems in the hierarchy or inappropriate management of animals are also pointed out as sources of stress (Lebrun et al. 2010, Goossens et al. 2016a). Stress causes reduction of peristalsis, leading to inadequate digestion and availability of nutrients that favor *C. perfringens* proliferation in the intestine (Allaart et al. 2013, Goossens et al. 2016a).

C. perfringens is a gram-positive, anaerobic, and spore-forming bacterium that is present in the environment and in the normal intestinal microbiota. However, situations of imbalance with an exacerbated proliferation of this bacterium are associated with diseases of the digestive tract. *C. perfringens* is a toxinogenic bacterium and its classification can be made according to the pattern of toxin production in type A, B, C, D, and E strains (Morris et al. 2012, Allaart et al. 2013, Verherstraeten et al. 2013, Goossens et al. 2016b, 2017). Isolation and genotyping of bacteria in the intestinal contents and feces of animals of this report evidenced only type A strains. This is consistent with the findings in animals with necrohemorrhagic enteritis, in which the isolates are almost exclusively from type A strains (Lebrun et al. 2010, Morris et al. 2012, Allaart et al. 2013, Goossens et al. 2016a, 2016b, 2017). However, there are controversies regarding the role of *C. perfringens* type A strains and their toxins in the pathogenesis of the disease.

Alpha toxin is the major toxin produced by type A strains. Studies have pointed out that alpha toxin is involved in the induction of lesions in the intestine, but its action may depend on the amount it is produced (Lebrun et al. 2010, Morris et al. 2012, Goossens et al. 2016a). Another theory states that this toxin triggers necrohemorrhagic enteritis by synergism with other toxins, such as a beta-2 (Lebrun et al. 2010) and perfringolysin O (Verherstraeten et al. 2013). Goossens et al. (2017) proposed that necrohemorrhagic enteritis occurs when there is an orchestrated action of toxins and enzymes produced by bacteria and their association with predisposing factors necessary to trigger the disease in the animal (Goossens et al. 2016a). This is consistent with the scenario observed in this



Fig.1. Small intestine, bovine. (**A**) Edematous, hemorrhagic small intestine distended by large amounts of gas within it. (**B**) Mucosa of the small intestine diffusely necrotic and hemorrhagic, as well as showing hemorrhagic streaks (vibices).

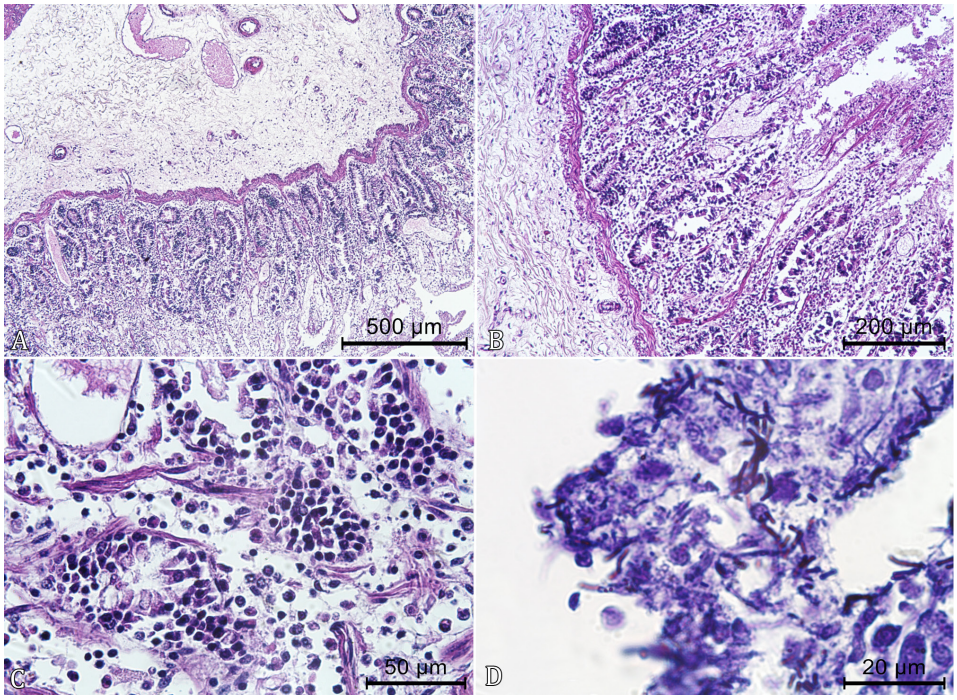


Fig.2. Small intestine, bovine. (**A**) Edematous submucosa with blood vessels filled with blood and discrete inflammatory infiltrate. HE, obj.5x. (**B**) Mucosa of the small intestine presenting villus necrosis and degeneration of crypt epithelium, in addition to a marked inflammatory infiltrate. HE, obj.10x. (**C**) Inflammatory infiltrate in the mucosa of the small intestine predominantly composed of mononuclear and rare polymorphonuclear cells. HE, obj.40x. (**D**) Gram-positive bacilli in the mucosa of the small intestine. Gram, obj.100x.

report, in which *C. perfringens* type A and positive for beta-2 encoding gene were isolated from feces and intestinal contents of animals found dead.

However, because *C. perfringens* type A strains are found in the intestinal microbiota of healthy animals, both bacterial isolation and toxin detection can be performed in the feces of healthy animals and hence have a little diagnostic value alone (Goossens et al. 2016a). Strains of *C. perfringens* are found at levels of 10^2 to 10^5CFU/g of intestinal content in healthy animals. Higher counts, ranging from 10^6 to 10^8CFU/g, are related to animals with necrohemorrhagic enteritis. The best material for isolation, which has the highest amount of bacteria and reflects the exacerbated proliferation of clostridia, is the intestinal content from areas with necrohemorrhagic lesions (Lebrun et al. 2010). This explains why fecal samples showed counts compatible with healthy animals, while intestinal contents showed counts higher than 10^6 CFU/g, confirming the diagnosis of necrohemorrhagic enteritis. However, there is recent evidence that animals with the disease may have normal counts of *C. perfringens* type A even in intestinal contents. What is claimed is that predisposing factors provide an environment in which toxin production is regulated positively. This suggests that an increased toxin production and lesion development are not directly related to the exacerbated proliferation of the etiological agent (Goossens et al. 2016a).

Regarding the macroscopic pattern of lesions, all the 57 animals exhibited lesions characteristic of necrohemorrhagic enteritis, including inflamed small intestine, distended due to gas accumulation, hemorrhagic and necrotic mucosa, abundant bloody fluid content, and rapid putrefaction of tissues in cadavers. In relation to the histopathological findings, mucosal layer with diffuse necrosis of the villi in association with a hemorrhagic content and marked inflammatory mononuclear and polymorphonuclear infiltrate are commonly described. Degeneration of the myenteric plexus and submucosal plexus may also be present (Lebrun et al. 2010, Goossens et al. 2016a), being only the first observed in this report.

CONCLUSION

On basis of the epidemiological data and findings from anatomopathological exams, an outbreak of necrohemorrhagic enteritis was determined. Adding tests of bacterial isolation and PCR genotyping, it was possible to find *Clostridium perfringens* type A as the etiological agent involved, with positivity for beta-2 toxin encoding gene. As showed, this condition can cause significant losses if food mismanagement of herds is present, with healthy animals evolving acutely to death.

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

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Experimental infection by *Anaplasma marginale* in buffaloes and cattle: clinical, hematological, molecular and pathological aspects[1]

Danillo H.S. Lima[2]*, Wagner M.S. Vinhote[2], Daniel G. Ubiali[6], Pierre C. Soares[7], Matheus D. Cordeiro[5], Jenevaldo B. Silva[3], Adivaldo H. Fonseca[5] and José D. Barbosa[4]

ABSTRACT.- Lima D.H.S., Vinhote W.M.S., Ubiali D.G., Soares P.C., Cordeiro M.D., Silva J.B., Fonseca A.H. & Barbosa J.D. 2019. **Experimental infection by *Anaplasma marginale* in buffaloes and cattle: clinical, hematological, molecular and pathological aspects**. *Pesquisa Veterinária Brasileira 39(9):700-709*. Faculdade de Medicina Veterinária, Instituto de Medicina Veterinária, Campus de Castanhal, Universidade Federal do Pará, Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mail: danillo.lima@ifpa.edu.br

The study aimed to evaluate and compare the clinical, laboratory and pathological aspects of buffalo and bovine experimentally infected with AmRio 2 strain of *Anaplasma marginale*. Four Murrah buffaloes and four crossbred cattle were used in the experiment, which two animals of each species were splenectomized. Strain AmRio 2 of *A. marginale* was inoculated in all experimental animals. Clinical exams, Packed Cell Volume (PCV), blood counts, blood smears, rickettsemia, necropsy and histopathology were performed in all cases. Semi-Nested-PCR (snPCR) for the *msp5* and snPCR for the *msp1α* target gene for identification of *A. marginale* in blood samples from animals was done. From positive samples for *msp1α* snPCR, samples were analyzed for the amino acid sequences of this gene. Two splenectomized cattle presented apathy, pale mucous membranes, jaundice, hyperthermia, and severe anemia. The remaining experimental animals did not show clinical signs. The rickettsemia in all animals was less than 1%. The mean PCV of the splenectomized cattle was below 20% at two-time points after infection. On the blood count, the main changes were observed in splenectomized calves and were characterized by a decrease in red blood cells, hemoglobin, PCV and platelets ($p < 0.05$). All animals presented leukocyte elevation by increased lymphocytes, however, with no significant difference. The average prepatent period was two days in all the animals. The average incubation period in cattle that became ill was 25.5 days, and death occurred, on average, 63 days after inoculation of the strain. The necropsy findings were characterized by pale carcass, ascites, enlarged liver, distended gallbladder, and thick bile. Histopathological findings included infiltration of macrophages and lymphocytes in various organs, hepatic sinusoidal dilatation, and necrosis of the large intestine. In snPCR for the *msp5* gene, 100% of the animals were positive in at least one evaluation. And in the snPCR for the infection of the *msp1α* target gene was also found in all animals in at least one sample evaluated.

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[2] Instituto Federal do Pará (IFPA), Campus Rural de Marabá (CRMB), Rodovia BR-155 Km 25, Marabá, PA 68508-970, Brazil. E-mail: marcelo.vinhote@ifpa.edu.br; *Corresponding author: danillo.lima@ifpa.edu.br

[3] Instituto de Ciências Agrárias (ICA), Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Av. João Narciso 1380, Bairro Cachoeira, Unaí, MG 38610-000, Brazil. E-mail: jenevaldo@hotmail.com

[4] Faculdade de Medicina Veterinária, Instituto de Medicina Veterinária, Campus de Castanhal, Universidade Federal do Pará (UFPA), Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mails: diomedes@ufpa.br

[5] Laboratório de Doenças Parasitárias, Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural do Rio de Janeiro (UFRRJ), Rodovia BR-465 Km 7, Seropédica, RJ 23890-000, Brazil. E-mails: adivaldofonseca@yahoo.com, mathcordeiro@hotmail.com

[6] Departamento de Epidemiologia e Saúde Pública, Instituto de Veterinária (IV), Universidade Federal do Rio de Janeiro (UFFRJ), Seropédica, RJ 23890-000, Brazil. E-mail: danielubiali@hotmail.com

[7] Departamento de Medicina Veterinária, Universidade Federal Rural de Pernambuco (UFRPE), Rua Dom Manuel de Medeiros s/n, Bairro Dois Irmãos, Recife, PE 52171-900, Brazil. E-mail: pcastro.pe@gmail.com

However, sequencing revealed only five animals, including the bovine which died, with a similarity of the amino acid sequences with AmRio 2 strain of *A. marginale*. It is concluded that the splenectomized cattle died due to anaplasmosis caused by the inoculated strain and the buffalo were more resistant compared to cattle. Buffaloes can be an alternative to cattle rearing in areas with a high occurrence of clinical cases of anaplasmosis.

INDEX TERMS: Experimental infection, *Anaplasma marginale*, buffaloes, cattle, clinics, hematology, molecular aspect, pathology, anaplasmosis, calves, AmRio 2 strain, clinical signs, PCR, bacterioses.

RESUMO.- [Infecção experimental de *Anaplasma marginale* em búfalos e bovinos: aspectos clínicos, hematológicos, moleculares e patológicos.] O estudo teve como objetivo avaliar e comparar os aspectos clínicos, laboratoriais e patológicos de búfalos e bovinos infectados experimentalmente com estirpe AmRio 2 de *Anaplasma marginale*. Para isso, foram utilizados quatro bubalinos Murrah e quatro bovinos mestiços, sendo dois animais de cada espécie, esplenectomizados. Estirpe AmRio 2 de *A. marginale* foi inoculada em todos os animais. Foram realizados exames clínicos, hematócrito, hemograma, esfregaço sanguíneo com avaliação de riquetsemia, necropsia e histopatologia, além de, Semi-Nested-PCR (snPCR) para o gene alvo *msp5* e snPCR para o gene alvo *msp1α* para identificação de *A. marginale* nas amostras de sangue dos ruminantes. A partir das amostras positivas na snPCR *msp1α*, foram selecionadas amostras para análise das sequências de aminoácidos deste gene. Dois bovinos esplenectomizados apresentaram apatia, mucosas pálidas, icterícia, hipertermia e anemia severa. O restante dos animais não apresentou sintomatologia clínica. A riquetsemia em todos os animais foi menor que 1%. A média do hematócrito dos bovinos esplenectomizados esteve abaixo de 20% em dois momentos após infecção. Ao hemograma, as principais alterações observadas foram nos bovinos esplenectomizados e caracterizaram-se por redução de hemácias, hemoglobina, hematócrito e plaquetas ($p<0,05$). Todos os animais apresentaram elevação de leucócitos por aumento de linfócitos, porém, sem diferença significativa. O período pré-patente médio foi de dois dias em todos os animais. O período de incubação médio nos bovinos que adoeceram foi de 25,5 dias e estes morreram em média 63 dias após inoculação da estirpe. Os achados de necropsia caracterizaram-se por carcaça pálida, ascite, aumento de volume do fígado, vesícula biliar distendida e bile espessa. À histopatologia, verificou-se infiltração de macrófagos e linfócitos em diversos órgãos, dilatação dos sinusoides hepáticos e necrose do intestino grosso. A snPCR para o gene *msp5*, revelou 100% dos animais positivos em pelo menos um momento de avaliação. E na snPCR para o gene alvo *msp1α* também verificou-se infecção em todos os animais em pelo menos uma amostra avaliada. Entretanto, o sequenciamento revelou apenas cinco animais, incluindo os bovinos que morreram, com similaridade das sequências de aminoácidos com estirpe AmRio 2 de *A. marginale*. Conclui-se que os bovinos esplenectomizados morreram em virtude de anaplasmose provocada pela estirpe inoculada e os bubalinos foram mais resistentes em comparação aos bovinos. Finalmente, os búfalos podem ser uma alternativa à criação de bovinos em áreas com alta ocorrência de casos clínicos de anaplasmose.

TERMOS DE INDEXAÇÃO: Infecção experimental, *Anaplasma marginale*, búfalos, bovinos, clínica, hematologia, aspecto molecular, patologia, anaplasmose, bezerros, estirpe AmRio 2, sinais clínicos, PCR, bacterioses.

INTRODUCTION

Anaplasma marginale is an obligate intracellular bacterium. It can be biologically transmitted by ticks, and mechanically transmitted by hematophagous dipterans, infected blood in photites, and also transplacental (Aubry & Geale 2011, Grau et al. 2013). This rickettsia causes a condition called anaplasmosis, which is endemic in tropical and subtropical areas, and result in considerable economic loses for the beef and dairy industries worldwide (Kocan et al. 2015).

Anaplasmal cattle may present fever, weight loss, abortion, lethargy, jaundice, and often death mainly of animals over two years of age (Kocan et al. 2003). Buffaloes present with lack of appetite, depression, ruminal atony, jaundice, pale mucous membranes (Srivastava & Ahluwalia 1974, Reddy et al. 1988), weakness, anorexia, tachycardia and difficulty in breathing (Vatsya et al. 2013).

Despite the confirmed presence of this rickettsia in buffalo herds (Silva et al. 2014a, 2014b, 2014c, 2015), there is little knowledge about the development of anaplasmosis in this species. Furthermore, there is no research that simultaneously evaluates the pathogenicity of *A. marginale* in cattle and buffaloes and compares the effects of the disease between the two species. This fact is relevant because buffaloes play an important role as reservoirs for *A. marginale*, serving as a source of infection for cattle when raised in the same location (Sharma 1987, Silva et al. 2014c). This situation is frequently observed in some regions of the state of Pará, especially in Marajó Island, which has the largest herd of buffaloes in Brazil (IBGE 2016).

Based on this, the present study aimed to evaluate and compare the clinical, laboratory and pathological aspects of buffaloes and experimentally infected cattle with AmRio 2 strain of *A. marginale* (Genbank: KM023771).

MATERIALS AND METHODS

Study location. The experiment was developed at the "Instituto Federal do Pará, Campus Rural de Marabá", (IFPA-CRMB) (5°33'44.5"S and 49°06'01.1"W), Marabá, Pará, Brazil. The study period took place from January to May 2017, when the daily averages of environmental conditions consisted of 26.1°C temperature, 91.3% relative humidity and 8.7mm rainfall. Data collected by the hydrometeorological station located at IFPA-CRMB.

Animals and handling. This study was approved by the Animal Use Ethics Committee under protocol number 2134171215 registered in December 2015.

We used four buffalos (*Bubalus bubalis*) of the breed Murrah and four crossbred cattle (*Bos taurus* × *Bos indicus*) with average age of 10 months, male, not castrated, and average weight of 148kg. Two animals of each species were splenectomized.

The animals were raised in two different environments at different times of the experiment. At first, they were separated by species and kept in two screened stalls, with a maximum number of four animals per stall. The food was based on *Brachiaria brizantha* cv. Marandu, 21% crude protein food (0.5kg/animal/day), mineral salt (40g/animal/day) and water as needed. Then, the animals were raised on *B. brizantha* cv. Marandu.

Since the animals came from anaplasmose endemic regions, we performed semi-nested PCR (snPCR) before the experiment and verified that the animals were positive for *Anaplasma marginale*. Therefore, 160 days before the beginning of the experiment, the animals were treated with imidocarb dipropionate, 5mg/kg subcutaneously, single application and long-acting oxytetracycline, 20mg/kg intramuscularly, three times, 48 hours apart. Fifteen days after treatment, we performed an snPCR and verified that two bovines not splenectomized were still infected by a native strain of *A. marginale*. Thus, 65 days before rickettsia inoculation, we applied a second treatment on all animals using the above-mentioned drugs and doses. However, only non-splenectomized cattle remained positive in two snPCRs performed at weekly intervals between blood samples. These animals came from the same state, where there was a history of clinical cases of anaplasmosis in calves. Nevertheless, we decided to maintain these two cattle in the experiment due to the difficulty in obtaining negative animals for *A. marginale* in the region.

All animals were bathed with 12.5% amitraz at a dilution of 20ml in 10l of water and 2.5l dose of mixture/animal; we sprayed 15% cypermethrin into the environment at the dilution of 20ml in 20l of water and 10l dose of the mixture/well twice, with 21 days apart in both treatments.

Experimental design. Four buffaloes (two non-splenectomized and two splenectomized) and four cattle (two non-splenectomized and two splenectomized) were inoculated with AmRio 2 strain of *A. marginale* (Baêta et al. 2015). We performed periodical clinical examinations, hematocrit blood samples, blood count and blood smear with rickettsemia evaluation to verify the infection and the possible development of anaplasmosis in the studied animals. We performed necropsy in the dead animals, collected samples and performed histopathological exam. We also performed molecular analysis for the identification of *A. marginale* using Semi-Nested PCR (snPCR) with the animals' blood samples.

The experiment was divided in two phases. In the first, the ruminants remained for 30 days in screened pens. The aim was to evaluate and describe the clinical changes more carefully. In the second, the ruminants were remained for 58 days in paddocks. This phase aimed to evaluate, in a larger chronological space, the occurrence of anaplasmosis in experimental animals and a possible occurrence of genetic diversity of the AmRio 2 strain of *A. marginale.*

Inoculation of AmRio 2 strain of *Anaplasma marginale.* The inoculum used for application to animals was the AmRio 2 strain of *A. marginale*, kept in culture of embryonic *Ixodes escapularis* tick cells (strain IDE8) (Baêta et al. 2015). The inoculum was prepared from a culture with a percentage of infection higher than 70%, evaluated by cytocentrifuge smears under optical microscope (Olimpus®). Upon observation of the smear and confirmation of the minimum infection value, the inoculum vials were rinsed inside the laminar flow using a sterile Pasteur pipette (Kasvi®). Subsequently, the cell media was transferred to a curved needle syringe and then discarded in sterile beaker. The medium contained in the beaker was transferred in the volume of one (1) mL to three (3) mL syringe with 40 × 12mm needle for inoculation in animals, which was performed through the jugular vein.

Clinical examination. Clinical examination was performed according to Dirksen et al. (1993) to verify rectal temperature (RT), heart rate (HR), respiratory rate (RR), ruminal movements (MR), mucosal staining, lymph node size and behavior. Physiological parameters were obtained by daily clinical examinations 20 days before starting the study. And the means obtained in these tests were parameters for comparison with the data obtained after the rickettsia inoculation.

After inoculation, clinical examinations were performed from D0 (day of inoculation) to D30 in the morning, once a day, during the animals' stay in the pens. Temperature was measured with a digital thermometer twice a day, with an average interval of 12 hours between measurements. From the moment the animals started being raised on paddocks, they were observed daily for behavior, food and water intake and clinical examinations, as well as temperature measurements, performed once a week. We used previously described values as standard values for cattle (Dirksen et al. 1993) and buffaloes (Moraes Júnior et al. 2010).

Hematological and parasitological evaluation. We collected blood samples for blood count, manual hematocrit and blood smear once a week for 30 days before the start of the experiment. The averages obtained in this period constituted the values before inoculation, which were considered as control. From the inoculation, for blood count, blood samples were collected in D7, D14, D21, D28 and D48 through the jugular vein, with the aid of a vacutainer system. The samples were packed in tubes with EDTA (Ethylenedeamine Tetra Acetic Acid), which were taken to a commercial laboratory located in Marabá, Pará, where they were routinely processed in BC-2800Vet® device (Shenzhen Mindray Bio-Medical Eletronics®, Germany) and the reference standards adopted were those described by Jain (1993) for cattle and Abd Ellah et al. (2014) for buffaloes. For the purpose of analysis of hematocrit results, only the results of the manual technique were used, disregarding the values obtained in the automated technique.

Manual hematocrit and rickettsemia evaluation were performed in D2, D5, D7, D9, D12, D14, D16, D19, D21, D26, D28 (period in the stalls), D35, D42, D48, D56, D60 and D70 (period in the pasture). The hematocrit was performed according to the specifications of Thrall et al. (2007). Blood smears were performed using a commercial kit (Instant-Prov®) for fixing and staining. The prepared slides were analyzed under a light microscope with a 100× objective and 10× eyepiece, in immersion oil. Rickettsemia was obtained by visualizing 40 microscopic fields to calculate the percentage of infected red blood cells. Animals were considered positive when presenting ≥0.01% of infected red blood cells.

In parallel to blood sampling for hematocrit, blood smear and rickettsemia, snPCR scans were performed by analysis of the genes *msp5* and *msp1a* of the AmRio 2 strain of *A. marginale*.

Necropsy and histopathology. During necropsies of two animals we collected several organs, which were stored in 10% buffered formaldehyde and sent to the "Setor de Anatomia Patológica" (SAP) of the "Universidade Federal Rural do Rio de Janeiro" (UFRRJ), where they were submitted to histopathological routine tests, cut into 5µm and stained with hematoxylin and eosin (HE).

DNA extraction. DNA samples were extracted from 200µl of animal blood using the protocol described by Sambrook & Russel (2001). We added to the samples 20µl Proteinase K (20mg/mL) and 200µl digestion buffer (20mM Tris-HCl, 20mM EDTA, 400mM NaCl, 1% Sodium Dodecyl Sulphate and 10mM CaCl2). After homogenizing the sample by inversion, it was incubated at 56°C overnight. DNA extraction was started with the addition of 460µl chloroform,

vigorous homogenization and subsequent addition of 240µl of protein precipitation solution (3M potassium acetate, 11% glacial acetic acid). After homogenization, the mixture was centrifuged at maximum speed for 10min. At the end of centrifugation, the aqueous portion was transferred to a microcentrifuge tube containing 700µl of cold isopropanol for DNA precipitation. The total DNA precipitate was centrifuged at maximum speed for 4min. The supernatant was discarded, and the formed pellets were washed twice with 1mL cold absolute ethanol and 70% alcohol at full speed for 2min. The pellets were oven dried at 56°C for 15min. Then the DNA was rehydrated with 100µl TE 1X at 56°C for 1 hour and stored in a freezer until testing was performed.

snPCR for target genes *msp5* e *msp1α*. For screening of positive and negative animals, snPCR was performed with the Amar primers *msp5* eF (5'GCATAGCCTCCGCGTCTTTC 3'), Amar *msp5* iF (5'TACACGTGCCCTACCGAGTTA 3') and Amar *msp5* eR (5'TCC TCG CCTTGGCCCTCAGA 3'), previously described by Torioni de Echaide et al. (1998) and optimized by Singh et al. (2012). The first reaction was performed with the primers eF and eR, and the second reaction used the primers iF and eR. The reactions were performed in a thermal cycler (Bio Rad T100™) with the following conditions: Polymerase activation at 95 °C for 5min, initial denaturation at 94°C for 1 min, annealing at 58°C for 1min, extension at 72°C for 1min (35 cycles), final extension at 72°C for 5min.

In the first reaction the final volume of the mixture was 25µl, consisting of 15.55µl of ultrapure water, 0.2µl of Taq polymerase (5U/µL), 0.5µL of each primer (10pmol), 2µL of DNTP (2.5mM), 0.75µL of Mg (50mM), 2.5µL of buffer (10X) and 3µL of DNA. In the second reaction, the quantities were similar except for the volume of ultrapure water, which was 16.55µL and the added volume of one microliter of PCR product from the first reaction.

The snPCR *msp5* positive samples were selected for evaluation of the *msp1α* gene for strain differentiation in the positive animals. These samples were selected on random collection days so that all animals were evaluated at least one day. Thus, snPCR was performed with the primers 1733F (5'-TGTGCTTATGGCAGACATTTCC-3'), 3134R (5'-TCACGGTCAAAACCTTTGCTTACC-3') and 2957R (5-'AAACCTTGTAGCCCCAACTTATCC-3') for the target gene *msp1α* (Lew et al. 2002). The reaction was performed under the following conditions: initial denaturation at 94°C for 4min, 35 cycles at 94°C for 30 seconds, 55°C (60°C second reaction) at 1min and 72°C at 2min followed by an extension final at 72°C for 7min. The first and second reactions were made with the final volume of the 25µl mix with the reagents at the same concentrations described in the snPCR above. SnPCR products were analyzed on 1.5% agarose gel stained with ethidium bromide. Running time was 90min, 80V and intensity 160.

Sequencing of the *msp1α* gene in *Anaplasma marginale*. Molecular identification of the AmRio 2 strain was performed by sequencing fragments of the A. marginale *msp1α* gene obtained from snPCR. The fragments were purified with ExoSAP-IT® PCR (USB®) and sequenced by the Sanger method in DNA ABI 3730 Sequencer (Applied Biosystems, Life Technologies®). The generated sequences were compared to data published using NCBI Nucleotide BLAST.

Statistical analysis. Initially data were described by means and means of standard error and were tested for distribution normality using the Kolmogorov-Smirnov test. Variables that did not meet the normality assumptions were submitted to logarithmic transformation (log10) or square root [RQ (X + 1/2)]. Then, the data were submitted to analysis of variance (ANOVA). When the ANOVA F test was significant, the contrast was averaged by the least significant difference (d.m.s.) of the Student-Newmn-Keuls test at 5% probability (p=0.05). For the variables analysis, we used the procedure "General Linear Model" (GLM) of the statistical program "Statistical Analysis System" (SAS 2009).

RESULTS

Clinical, hematological and parasitological aspects

Two splenectomized cattle (Cattle 3 and 4) presented anaplasmosis at different times. Cattle 3 presented clinical alterations on the 9th day after infection (PID), which were characterized by apathy, decreased appetite, slight mucosal pallor, pasty feces, mean RT 39.8°C, hematocrit 15% and 1% red blood cells infected with *Anaplasma marginale* corpuscles in the blood smear (Fig.1A). This animal spontaneously recovered in two days, confirmed by the absence of clinical signs and return of hematocrit to 25%. However, the patient had recurrence in the 60th PID and had no appetite, poor body score (Fig.1B), sialorrhea, whitish ocular mucosa (Fig.1C), mild jaundice, evident tachycardia, ruminal and intestinal atony, TR of 40°C, faecal stools (Fig.1D), some with mucus and blood streaks, 8% hematocrit and countless *A. marginale* inclusion corpuscles within red blood cells. This animal was treated with long-acting oxytetracycline, 20mg/kg, 30% (5ml) intravenously and 70% (13ml) intramuscularly, three times, 24 hours apart; imidocarb dipropionate, 5mg/kg subcutaneously, single application; blood transfusion (about 700ml of donor heifer blood) and fluiodotherapy (1l of 0.9% saline plus 500ml of vitamin complex). Eleven days later, the hematocrit returned to 25% and the animal had no clinical symptoms. However, after 19 days of treatment, the animal again presented symptoms more intensely described above and died. Cattle 4 presented 20% of hematocrit and countless inclusion corpuscles of *A. marginale* in the 42nd PID. Two days after hematocrit spontaneously returned to 26%. However, at D46 the animal had mild apathy, faecal stools and mean RT of 39.7°C, and died the next day.

An individual animal overview of the clinical and parasitological aspects is presented in Table 1. Regarding the means of RT when compared to values before inoculation, except for non-splenectomized buffaloes, the groups presented high RT during the whole evaluation period after inoculation of *A. marginale* strain AmRio 2, but with values within the normal reference range for the species. Only splenectomized cattle showed febrile peaks during the experiment (Fig.2). The hematocrit average of splenectomized cattle was below the values before inoculation and reference for the species in D9 and D60. Splenectomized buffaloes had a mean hematocrit below the values before inoculation and near the lower limit of the reference value for the species in D2 and D7. (Fig.3).

Cattle and buffalo blood count values are described in Tables 2 and 3, respectively. Both groups of buffaloes did not present alterations that differed statistically. However, we observed leukocyte elevation in these animals due to lymphocyte increase, when comparing the values before and after inoculation of the AmRio 2 strain of *A. marginale*. Despite the increase in white cells, the values remained within the reference range for the species (Abd Ellah et al. 2014).

As for cattle, the non-splenectomized group presented an increase in MCV with a statistical difference ($p<0.05$), and the splenectomized group presented a decrease in red blood cells, hemoglobin, platelets and an increase in MCV



Fig.1. Experimental infection with AmRio 2 strain of *Anaplasma marginale* in cattle and buffalos. (**A**) Cattle 3 blood smear with presence of *A. marginale* corpuscles in PID 9. (**B**) Cattle 3 showing apathy and severe weight loss at 60th PID. (**C**) Cattle 3 with pale left ocular mucosa. (**D**) Cattle 3 with recently obtained rectal-shaped stools.

Table 1. Clinical symptomatology, post-infection day (PID) of clinical change, mean frequency of blood smear infection, mean rickshaemia, days of higher levels of rickettsemia, and recovery of buffalo and cattle experimentally infected with AmRio 2 strain of *Anaplasma marginale*

Animal	Clinical symptomatology	PID	Blood smear (%)	Rickettsemia (%)	Days of higher levels of rickettsemia	Recovery
Cattle 1 (non-splenectomized)	No	-	47	0.73	D2 (4%); D21 (Uncountable)	-
Cattle 2 (non-splenectomized)	No	-	27	0.26	D2 (1%)	-
Cattle 3 (splenectomized)	Yes	9th and 60th	38	0.62	D2 (6%); D60 (Uncountable)	First, spontaneous. Second, with treatment. Then death.
Cattle 4 (splenectomized)	Yes	42nd e 46th	23	0.38	D2 (4%); D42 (Uncountable)	First, spontaneous. Then death.
Buffalo 1 (non-splenectomized)	No	-	33	0.80	D2 (Uncountable)	-
Buffalo 2 (non-splenectomized)	No	-	47	0.27	D5 (2%)	-
Buffalo 3 (splenectomized)	No	-	27	0.53	D2 (6%)	-
Buffalo 4 (splenectomized)	No	-	27	0.60	D2 (Uncountable); D5 (6%)	-

after pellet inoculation with rickettsia. Red blood cell and platelet values in this latter group were above the reference range (Jain 1993) even before inoculation.

In both groups of cattle, we also observed an increase in leukocytes due to elevation of lymphocytes after inoculation, but without statistical difference. The values remained at the reference limit in non-splenectomized cattle, whereas in splenectomized cattle the average lymphocyte count after inoculation was slightly above the reference level (Jain 1993).

The pre-patent period (PPP), based on blood smear, was two days in all animals except Buffalo 2, which was of five days. The average incubation period (PI) in the sick cattle was 25.5 days and these animals died on average 63 days after inoculation of the Amrio 2 strain of *A. marginale*.

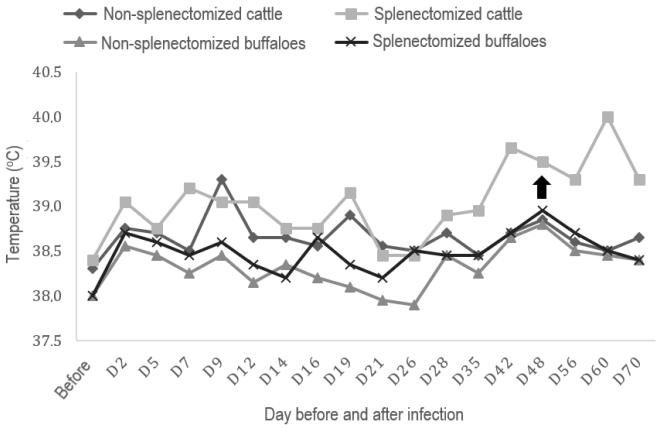


Fig.2. Mean rectal temperature (RT) values in buffalo and cattle before and after experimental infection with strain AmRio2 of *Anaplasma marginale*. From D48 (↑), in splenectomized cattle, the values correspond only to the results of Cattle 3, due to the death of Cattle 4.

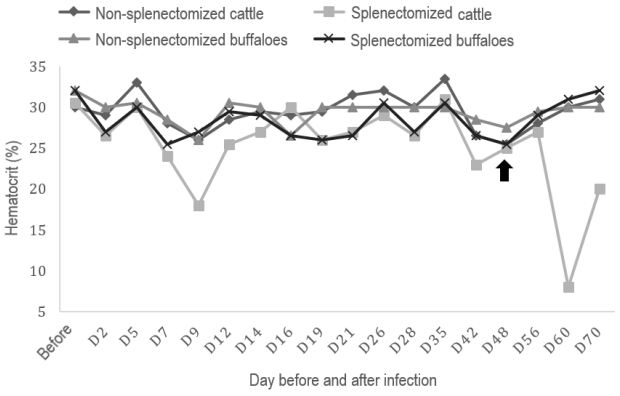


Fig.3. Mean hematocrit values in buffalo and cattle before and after experimental infection with strain AmRio2 of *Anaplasma marginale*. From D48 (↑), in splenectomized cattle, the values correspond only to the results of Cattle 3, due to the death of Cattle 4.

Table 2. Mean values, mean standard error and significance of buffalo blood count before and after experimental infection with AmRio 2 strain of *Anaplasma marginale*

Variables	Unit	Buffaloes			
		Non-splenectomized		Splenectomized	
		Before	After	Before	After
Red blood cells	x10^6/µl	7.80±0.42	8.13±0.23	8.33±0.11	8.11±0.17
Hemoglobin	g/dl	11.68±0.44	12.23±0.41	11.33±0.25	11.20±0.25
ACV*	fL	44.83±1.17	44.84±0.86	39.92±0.70	41.13±0.26
MCHC**	%	33.77±0.21	33.58±0.10	34.13±0.28	33.60±0.10
Platelets	x10^3/µl	414.96±53.11	285.20±28.57	659.38±52.21	579.60±30.30
Leukocytes	/µl	6862.50±1247.73	9292.00±1360.10	12762.50±931.26	14539.00±1171.76
Rods	/µl	61.75±17.76	124.20±27.95	123.63±11.15	208.20±48.12
Segmented	/µl	2641.25±287.55	2949.80±687.18	5334.50±899.14	2393.60±911.04
Lymphocytes	/µl	3824.50±860.61	5791.30±674.36	6841.38±767.10	9247.60±947.10
Eosinophils	/µl	72.38±26.38	165.50±97.10	43.63±28.42	200.80±65.91
Monocytes	/µl	165.63±31.59	264.60±41.67	389.50±111.10	400.60±67.71

* ACV = Average corpuscular volume, ** MCHC = mean corpuscular hemoglobin concentration. Absence of different lowercase letters on the same line indicates that there was no significant difference (p>0.05) within each group (non-splenectomized and splenectomized).

Table 3. Mean values, mean standard error and blood count significance of cattle before and after experimental infection by AmRio 2 strain of *Anaplasma marginale*

Variables	Unit	Cattle			
		Non-splenectomized		Splenectomized	
		Before	After	Before	After
Red blood cells	x10^6/µl	8.40±0.21	7.85±0.21	10.51±0.48^a	8.00±0.09^b
Hemoglobin	g/dl	11.05±0.26	11.40±0.31	12.11±0.70^a	10.48±0.09^b
ACV*	fL	38.85±0.58^b	43.00±0.91^a	37.70±0.51^b	39.12±0.66^a
MCHC**	%	34.03±0.43	33.94±0.19	34.43±0.77	33.58±0.08
Platelets	x10^3/µl	619.88±6.44	568.50±23.90	1845.13±269.38^a	1110.80±86.31^b
Leukocytes	/µl	7587.50±396.54	8487.00±904.53	9300.00±669.38	10357.00±803.62
Rods	/µl	59.13±19.84	84.90±9.05	74.38±24.93	103.60±8.04
Segmented	/µl	2630.00±406.67	2427.50±477.27	2208.05±274.17	2307.80±376.02
Lymphocytes	/µl	4394.63±447.31	5364.40±402.22	6546.63±944.89	7551.70±429.80
Eosinophils	/µl	107.75±22.95	324.20±192.76	96.00±48.10	70.30±11.21
Monocytes	/µl	251.38±25.84	260.50±16.85	327.13±43.65	323.60±34.87

* ACV = Average corpuscular volume, ** MCHC = mean corpuscular hemoglobin concentration. Lowercase letters on the same line indicates that there was no significant difference (p>0.05) within each group (non-splenectomized and splenectomized)

Necropsy and histopathology findings

Necropsy of both cattle revealed pale carcass, ascites, increased liver with rounded edges, full and distended gallbladder with thick bile, moderate jaundice and accumulation of dry stools in the rectum.

Histopathological analysis revealed moderate number of lymphocyte and mild macrophages infiltration into the heart, liver and kidney (Fig.4). Lymph nodes revealed histiocytic infiltration on the center of follicles. We also observed marked and diffuse hepatic sinusoid dilation, as well as diffuse cholestasis.

SnPCR for target genes *msp5* and *msp1α*

All animals on D0 were negative for *A. marginale* AmRio2 target gene *msp5*, except non-splenectomized cattle, which remained positive even with treatment before starting the study (Table 4). Positive animals were found to be more frequent from the time animals were raised on pasture (D35). Splenectomized cattle presented a higher average frequency of infection (50%) than spleen-free buffaloes (25%), and Buffalo 4 presented its first positive snPCR only two months after inoculation.

With the exception of Cattle 1 and 2 (non-splenectomized) and Buffalo 4 (splenectomized), during the period they remained in the stalls, there was considerable variation in the dynamics of infection, where animals detected as positive in one day did not necessarily continue the status in the following days.

The evaluation of the target gene *msp1α* revealed a similar result to that found in D0 in the analysis of the *msp5* gene. This confirmed that the animals were really negative at the beginning of the experiment, with execution of unsplenectomized cattle. On the following days, all samples selected for *msp1α* screening were positive at least one time.

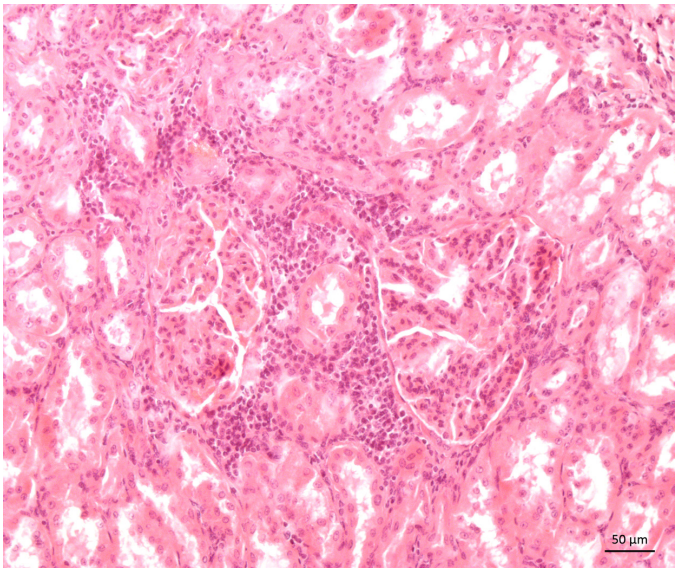


Fig.4. Experimental infection with strain AmRio 2 of Anaplasma marginale in buffaloes and cattle. Cattle 4 kidney with periglomerular lymphocytic infiltrate. HE, bar = 50µm.

Sequencing of *msp1α* gene in *Anaplasma marginale*

From the samples selected for sequencing, it was found that five animals (Cattle 2, 3 and 4; Buffalo 2 and 3) presented amino acid sequences (α β β β F) compatible with AmRio 2 strain of *A. marginale*.

DISCUSSION

In the present study, none of the buffaloes presented clinical signs of anaplasmosis, which differs from the findings of Sharma (1987) and Reddy et al. (1988) in experimental studies and those reported by Srivastava & Ahluwalia (1974) and Vatsya et al. (2013) in natural cases. These studies were developed in India and no report was found on experimental infection of *Anaplasma marginale* in buffalo in Brazil. Therefore, our research is the first to simultaneously compare several aspects of anaplasmosis in cattle and buffalo experimentally infected with *A. marginale* strain AmRio 2 in this country.

Only splenectomized cattle showed clinical signs of the disease, which corroborate those described by Lopes et al. (2016), Silvestre et al. (2016) and Aktas & Özübek (2017).

Table 4. Dynamics and frequency of infection with strain AmRio2 of *Anaplasma marginale* through Semi-Nested PCR for *msp5* gene in experimentally infected buffaloes and cattle as a function of animal blood collection in stalls (D0 - D28) and in pasture (from D28)

	D0	D2	D5	D7	D9	D12	D14	D16	D19	D21	D26	D28	D35	D42	D48	D56	D60	D70	Frequency
Cattle 1 (intact)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	94% (17/18)
Cattle 2 (intact)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100% (18/18)
Cattle 3 (splenectomized)	-	+	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	50% (9/18)
Cattle 4 (splenectomized)	-	+	-	-	-	-	-	+	-	+	+	+	+	+		Death			50% (7/14)
Buffalo 1 (intact)	-	-	-	-	-	-	-	+	-	+	-	-	+	+	-	+	+	+	39% (7/18)
Buffalo 2 (intact)	-	-	-	-	-	-	-	+	-	-	-	-	-	+	+	-	+	-	22% (4/18)
Buffalo 3 (splenectomized)	-	-	-	-	-	+	-	+	-	-	-	+	-	+	+	+	+	-	39% (7/18)
Buffalo 4 (splenectomized)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	11% (2/18)

+ Positive animal, - negative animal.

On the other hand, other experimental studies have reported mild clinical signs of anaplasmosis in splenectomized calves (Doyle et al. 2016a, Silvestre et al. 2016).

The frequency of positive animals for blood smear evaluation was similar between buffaloes (33.5%) and cattle (33.7%), as well as the mean values of rickettsemia in both buffaloes (0.55%) and cattle (0.50%). In general, the peak of rickettsemia in both species occurred in the 2nd PID. These findings differ from those verified by Reddy et al. (1988), who reported 8.5% of rickettsemia at the 29th PID in buffalo calves, and those reported by Gale et al. (1996), who found 2% to 34% of parasitized red blood cells with peaks between 12th to 24th PID in Hereford calves.

In the present study, two days of PPP and 25.5 days of PI were observed. Santos (2016) reported four-day PPP for *A. marginale* strain AmRio 1. According to Kocan et al. (2010), the PI can range from 7 to 60 days. The evolution of the clinical condition until the death of splenectomized cattle lasted on average 63 days and this shows that the strain presented high pathogenicity for this group.

Regarding temperature, only splenectomized cattle presented hyperthermia. This finding is in agreement with that verified by Silvestre et al. (2016), who observed fever of up to 40.2°C in experimentally infected cattle.

Marked changes were observed in splenectomized cattle for the manual hematocrit evaluation, which presented values below 15%, similar to that verified by Gale et al. (1996) and Lopes et al. (2016). On the other hand, non-splenectomized cattle and buffaloes, as well as splenectomized buffaloes, did not present significant changes in hematocrit, contrary to what was reported by Reddy et al. (1988) in buffaloes with spleen (20.6%).

Regarding the variables analysis on the erythrogram, both buffalo groups did not show any significant differences before and after inoculation. These data differ from those found in a case of natural infection by Vatsya et al. (2013). In relation to cattle, the alterations observed were greater in splenectomized animals, which were characterized by reduction of red blood cells, hemoglobin and platelets. The non-splenectomized cattle presented only increase of MCV. Doyle et al. (2016a) also reported a reduction in red blood cell count and hemoglobin concentration in splenectomized cattle.

Based on the white blood cell count, although not statistically significant, both non-splenectomized and splenectomized buffaloes and cattle showed elevation of leukocytes due to an increase in lymphocyte count, a change also reported by other authors (Doyle et al. 2016a, Silvestre et al. 2016). The decrease in red blood series elements is related to the decreased lifetime of red blood cells in the circulation caused by extravascular erythrophagocytosis due to the presence of rickettsia in the circulation (Massard et al. 1998). In relation to lymphocyte elevation, it probably represents an antigenic stimulation in response to *A. marginale* (Brown 2012).

Splenectomized animals had a high platelet count when compared to non-splenectomized animals of both species. However, when comparing the moments before and after inoculation, there was a decrease in the number of platelets in all groups evaluated. These results resemble those of Doyle et al. (2016b), who observed a significant decrease in platelets in splenectomized and intact cattle in the days after infection. Normally, up to one third of platelets are sequestered by the spleen (Randolph et al. 2010) and with surgical removal of this organ storage is impaired, which probably influenced the platelet values found in the present study. Transient or persistent thrombocytosis may also occur in asplenic humans (Sumaraju et al. 2001, Resende & Petroianu 2002).

The death of splenectomized cattle may be related to the greater susceptibility of these animals due to the absence of the spleen, as splenectomy impairs the formation of M immunoglobulins that appear at the beginning of the immune response (Klaus & Jones 1968). In contrast to cattle, buffaloes showed no clinical alteration. Therefore, it is suggested that buffaloes were more resistant when compared to cattle with respect to the strain evaluated in this study. In a similar study with *Babesia bovis*, Benitez et al. (2018) also observed that buffaloes did not show clinical symptoms and suggested that these animals had the ability to eliminate or significantly reduce circulatory hemorrhomas by an innate immunity mechanism.

Necropsy findings of splenectomized cattle were similar to those found in animals with anaplasmosis. According to Coetzee et al. (2005), these findings include severe anemia, jaundice, hepatosplenomegaly, petechial haemorrhages in the heart and pericardium, flabbiness and pallor of the heart. Histopathology revealed inflammatory process characterized by mononuclear cell infiltration into various organs. In addition, there was dilation of the liver sinusoids and cholestasis. These findings were similar to those described by Santos (1967).

Over 10 weeks of snPCR evaluation for the *msp5* target gene, there was a very variable infection dynamics, in which positive animals on one day did not necessarily continue their status on the following days. Animals that survive acute infection of *A. marginale* develop persistent infections characterized by low-level cyclic rickettsemia (Kieser et al. 1990, French et al. 1999).

Still on the snPCR for the *msp5* target gene, the non-splenectomized cattle started the positive experiment even after treatment against *A. marginale.* Based on sequencing, we found that these animals were infected with a native strain of this rickettsia. It is believed that the drugs used were not able to sufficiently reduce the amount of bacterial DNA in the bloodstream of animals to levels undetectable by snPCR. The rest of the animals responded to the treatment and started the snPCR negative search. When evaluating Table 4, the highest frequency of infection was at the time animals were on pasture. Two hypotheses may support this. The first, the animals came into contact with native strains of *A. marginale* from the moment they went to pasture, which contributed to a greater detection in snPCR for *msp5* gene, present in several *Anaplasma* species and used for serological and molecular screening studies (Torioni de Echaide et al. 1998, Bacanelli et al. 2014). The second, over time, the AmRio 2 strain increased the number of DNA copies in the circulatory current of the ruminants and, consequently, contributed to a higher sensitivity of the molecular examination and this can be supported by the fact that this time (pasture period) includes the period when rickettsemia increases (2 to 6 weeks after infection), in which levels of 10^9 or more bacteria per milliliter of blood can be verified by quantitative PCR (Futse et al. 2003, Han et al. 2010). The last hypothesis is the most likely.

In the comparative analysis of the snPCR result for *msp5* gene among splenectomized animals, cattle presented a significantly higher average frequency of infection than

buffaloes. Other studies also reported that buffaloes had lower values (Silva et al. 2014a, 2014b) compared to the observed in cattle (Bacanelli et al. 2014). A splenectomized buffalo was positive only 60 days after inoculation. It is likely that this animal had low rickettsemia in the period prior to the 60th PID, which possibly influenced the detection sensitivity of *A. marginale* DNA copies by *msp5* snPCR.

From the positive samples in the *msp5* gene evaluation, random animal samples were selected to be tested by *msp1α* target gene snPCR and 100% of the positive samples were found on at least one evaluation day. This indicates that the AmRio strain 2 actually caused infection in the animals, except in non-splenectomized cattle (Cattle 1 and 2), since they started the experiment positive for *A. marginale*. The fact that they were snPCR-positive for the *msp1α* gene may be related to the native strain in which these animals housed.

Despite the positive results observed in snPCR, analysis of the amino acid sequences based on the *msp1α* gene revealed that only five ruminants (Cattle 2, 3 and 4; Buffalo 2 and 3) showed similarity with *A. marginale* strain AmRio 2 tandem repeat sequence (α β β β F), as described first by Baêta et al. (2015). In these individuals it was possible to prove the infection by the strain under study. In the remaining animals (Cattle 1; Buffalo 1 and 4), the non-presentation of *A. marginale* AmRio2-like strain should be further investigated in future studies.

CONCLUSIONS

Blood smear and snPCR showed that AmRio 2 strain of *Anaplasma marginale* caused infection in experimentally infected buffalo and cattle. Based on the association of clinical, parasitological, histopathological and molecular findings, it was concluded that two splenectomized cattle died due to anaplasmosis caused by this strain.

Under the conditions of this experiment, buffaloes were more resistant to anaplasmosis than cattle, as they showed no relevant clinical and laboratory alterations after rickettsia inoculation.

According to our research data, buffaloes may be an alternative to raising cattle in areas with high frequency of clinical cases of anaplasmosis.

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Comparison of two collection methods to determine protodioscin concentrations in *Brachiaria* spp. and to monitor *Brachiaria* spp. poisoning[1]

Gleice K.A. Melo[2], Westerlly J. Silva[2], Rayane C. Pupin[3], Rubiane F. Heckler[2], Lucimara M. Nonato[2], Camila C.B.F. Ítavo[2], Valquíria B.N. Ferreira[4] and Ricardo A.A. Lemos[2]*

ABSTRACT.- Melo G.K.A., Silva W.J., Pupin R.C., Heckler R.F., Nonato L.M., Ítavo C.C.B.F., Ferreira V.B.N. & Lemos R.A.A. 2019. **Comparison of two collection methods to determine protodioscin concentrations in *Brachiaria* spp. and to monitor *Brachiaria* spp. poisoning**. *Pesquisa Veterinária Brasileira 39(9):710-714*. Laboratório de Anatomia Patológica, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul, Av. Senador Filinto Muller 2443, Campo Grande, MS 79074-460, Brazil. E-mail: ricardo.lemos@ufms.br

The objective of this study was to evaluate the methods of collecting *Brachiaria* spp. (Simulated Grazing vs. Total Collection) for the determination of protodioscin concentrations and to monitor the intoxication in sheep susceptible and resistant to poisoning by *Brachiaria* spp. The collection of *Brachiaria* spp. was done through two different methods. The first one consisted in throwing a metallic square at 10 random picket points. The second was performed simulating, with the hands, the grazing behavior of sheep. The leaf blades obtained by the two methods were conditioned in a forced ventilation oven. The extraction and quantification of protodioscin from the dry milled plant material was done by reverse-phase high-performance liquid chromatography (HPLC). The collections were carried out at three stages of the life of experimental lamb (suckling, rearing, and finishing). The lambs were divided into resistant and susceptible to intoxication by *Brachiaria* spp. The criterion for a lamb to be considered sensitive was to present some clinical signs at some stage of its life. No significant differences were observed between the general averages of the two collections when the evaluation was done in the whole period. However, a significant difference was found at the onset of the clinical signs. The concentrations of protodioscin in this period were higher in *Brachiaria* spp. collected by the simulating grazing method. Both methodologies were efficient to determine the level of protodioscin in the pastures of *Brachiaria* spp. However, during the periods when the outbreaks occurred, the simulated grazing method was more reliable.

INDEX TERMS: Collection methods, protodioscin concentrations, *Brachiaria* spp., poisoning, hepatogenic photosensitization, simulated grazing, toxic plants, small ruminants.

RESUMO.- [**Comparação entre dois métodos de coleta para determinar as concentrações de protodioscina em *Brachiaria* spp. e monitorar a intoxicação por *Brachiaria* spp.**] Objetivou-se avaliar dois métodos de coleta de *Brachiaria* spp. (Pastejo Simulado *vs* Coleta Total) para determinação das concentrações de protodioscina e efetuar o monitoramento da intoxicação em rebanhos sensíveis e resistentes à intoxicação por *Brachiaria* spp. Foram realizadas coletas de *Brachiaria* spp. através de duas metodologias. A primeira consistiu em fazer o lançamento de um quadrado metálico em 10 pontos aleatórios do piquete. A segunda foi realizada simulando com as mãos o comportamento de pastejo dos ovinos. As lâminas foliares obtidas pelas duas metodologias foram acondicionadas em estufa de ventilação forçada. O material seco e moído foi encaminhado para extração e quantificação da protodioscina por cromatografia líquida de alto desempenho (HPLC) de fase reversa. As coletas foram realizadas em três fases de

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[2] Graduate Studies Program in Animal Science, Faculdade de Medicina Veterinária e Zootecnia, Universidade Federal de Mato Grosso do Sul (UFMS), Av. Senador Filinto Muller 2443, Campo Grande, MS, 79070-900, Brazil. *Corresponding author: ricardo.lemos@ufms.br

[3] Graduate Studies Program in Veterinary Science, Faculdade de Medicina Veterinária e Zootecnia, UFMS, Av. Senador Filinto Muller 2443, Campo Grande, MS, 79070-900.

[4] Instituto Federal de Mato Grosso do Sul, Campus Aquidauana, Rua José Tadao Arima 222, Aquidauana, MS 79200-000, Brazil.

vida dos cordeiros (cria, recria e terminação). Os cordeiros foram divididos em resistentes e sensíveis a intoxicação por *Brachiaria* spp. O critério para o ovino ser considerado sensível foi apresentar algum sinal clínico da intoxicação em alguma fase de sua vida. Não foram observadas diferenças significativas entre a média geral das duas coletas quando a avaliação foi feita no período total. Porém, foi observada diferença significativa no período de aparecimento dos casos. As concentrações de protodioscina neste período foram superiores pelo método do pastejo simulado. Ambas as metodologias mostraram-se eficientes para determinação da concentração da protodioscina nas pastagens de *Brachiaria* spp. No entanto, nos períodos em que ocorreram os surtos, o método do pastejo simulado foi mais fidedigno.

TERMOS DE INDEXAÇÃO: Métodos de coleta, protodioscina, *Brachiaria* spp., intoxicação, fotossensibilização hepatógena, pastejo simulado, plantas tóxicas, pequenos ruminantes.

INTRODUCTION

Brachiaria spp. grasses are the main forages used in the central-western and southeastern regions of Brazil for ruminant forage (Porto et al. 2013), but are potentially toxic and cause economic losses due to death and reduced productive performance (Riet-Correa et al. 2011, Mustafa et al. 2012, Porto et al. 2013, Pupin et al. 2016). *Brachiaria* toxicity is due to lithogenic steroidal saponins contained in this grass and protodioscin has been the main saponin found in *Brachiaria* spp. (Riet-Correa et al. 2011). There are no well-defined methodologies for harvesting *Brachiaria* for determining protodioscin concentrations in this forage. There are reports of the levels of protodioscin determination in *Brachiaria* collected from different parts of the plant during an outbreak of poisoning in sheep, (Brum et al. 2007). In another study, protodioscin concentrations were evaluated at 28-day intervals according to the maturation stage of the plant (Faccin et al. 2014). However, the methodologies used did not take into account the grazing habits of sheep.

Considering that sheep consume the young leaves in preference over the senescent stems and leaves, that is, the whole forage canopy, and that the selected forage has different chemical and anatomical characteristics from the herbage available in the pasture (Goes et al. 2003, De Paula et al. 2009), the use of simulated grazing is the most representative method for collection when more reliable information is sought (Weir & Torell 1959, Cook 1964, Goes et al. 2003). This collection methodology could be applied with advantages over those previously used to evaluate protodioscin concentrations in *Brachiaria* spp.

The objective of this study was to compare two harvesting methodologies to determine the concentrations of protodioscin and simultaneously monitor the clinical course of the poisoning in *Brachiaria*-susceptible and *Brachiaria*-resistant lambs poisoned at three stages of their development.

MATERIALS AND METHODS

The experiment was carried out at the in the municipality of Campo Grande (20°44'71.95 "S 54°72'26.15'W, altitude 528m), state of Mato Grosso do Sul (MS) Brazil. The meteorological data were obtained from the National Institute of Meteorology (INMET 2015)/Center for Monitoring Climate and Water Resources of MS (Cemtec-MS)/Agency for Agrarian Development and Rural Extension (Agraer).

Collections of *Brachiaria* spp. were done from May to December, 2014 and from January to February, 2015 to determine the concentrations of protodioscin. The collections were performed every 28 days, using two different methods. The first one (total collection) consisted in casting a metallic square of 0.25m^2 (0.5x0.5m) in 10 random points representative of the forage canopy (McMenniman 1997) while sheep were grazing. The cuts were carried out close to the ground with the aid of metallic scissors. After cutting of all aerial parts, the samples were weighed individually, and then the plant material was homogenized and quartered, in order to obtain a representative sample from the picket. Subsequently, the morphological separation of the leaf blade, stem + sheath and senescent material (leaf blades and stems with progressive yellowing, visible darkening, and dehydration, characterizing the senescence phase of the tiller) were performed.

The second method consisted in simulate manually the movements of a sheep grazing (simulated grazing). The grazing behavior of the lambs were observed to determine the height and parts of the plant consumed, in order to obtain samples similar to those consumed by sheep. The grasping of the grass by the animal was reproduced with the hands, and 100 grams of the sample cut with the aid of a pocketknife (Cook 1964).

The leaf blades collected by the two methodologies were conditioned in a forced ventilation oven at 55°C for 72 hours, crushed in a Willey mill with 1mm mesh sieves and analyzed for dry matter (% DM). The extraction and quantification of protodioscin by reverse-phase high-performance liquid chromatography (HPLC) was performed according to a previously described methodology (Ganzera et al. 2001).

The plant samples were collected in pastures with a history of intoxication by *Brachiaria* spp. in sheep. During the experimental period, two groups were simultaneously observed for intoxication. The first was identified as sensitive, initially formed by two sheep and one ram. The second group was identified as resistant and consisted initially of three sheep and one ram. Sheep presenting clinical signs of *Brachiaria* spp. poisoning at some stage of their lives were considered sensitive. The resistant sheep were those that presented no clinical signs of *Brachiaria* spp. poisoning at any stage of their lives (Pupin et al. 2016).

The progeny of these two groups, 9 sensitive and 4 resistant lambs, were allocated in the same pickets, where the samples were collected. The experimental period was divided according to the life stage of the lambs, described as suckling, rearing and finishing. During the suckling stage, from May to July, 2014, the lambs and their mothers remained in continuous grazing on pickets formed by *Brachiaria brizantha*. On August 2014, the lambs were weaned and transferred to a picket containing *Brachiaria decumbens*, remaining there (rearing stage) in continuous grazing management until November 2014. The finishing stage began on December 2014, when the sheep entered in their reproductive life, remaining under the same management of continuous grazing in the pastures of *B. decumbens*. Clinical manifestations and *Brachiaria* collections were monitored until February 2015. Clinical course, necropsy and histopathological findings caused by *Brachiaria* spp. poisoning in sheep of the current study are described elsewhere (Pupin et al. 2016).

The Mann-Whitney test at 5% significance compared the results of the protodioscin values obtained by the two applied methodologies o plant collection.

RESULTS

The concentration of protodioscin (% dry matter - DM) in the samples collected through the selective grazing method ranged from 0.42 to 1.81% (1.020±0.448). In plant material sampled by total collection, the concentration ranged from 0.24 to 1.22% (0.773±0.328). There was no significant difference between the general means of the two sampling methods by the Mann-Whitney test (P>0.05) when the evaluation was made for the whole experimental period. However, a significant difference (P<0.05) was observed by the same test from July to November of 2014 (Table 1).

Cases of intoxication were observed in the lambs of the sensitive herd. Of the nine lambs monitored, eight presented clinical signs of intoxication by *Brachiaria* spp. After reintroduction of the lambs in the pastures, clinical recurrences were detected and the lambs were again hospitalized (Table 1). The four lambs of the resistant herd were monitored daily during the three phases of the experimental period and none of them presented clinical signs of intoxication.

In the suckling stage (May-July, 2014), no cases of poisoning were observed in lambs in *B. brizantha* pastures. However, clinical signs of Brachiaria poisoning, including relapses, were observed in all months of the rearing stage (August-November, 2014). In the finishing stage (December 2014-February, 2015) only one relapse was recorded (Table 1).

In the suckling stage (May-July, 2014), when no lamb showed clinical signs of *Brachiaria* poisoning, protodioscin concentrations were similar between selective grazing and total collection. Likewise, in the termination phase when a relapse was recorded in February 2015, protodioscin concentrations did not differ between the methodologies studied (Table 1).

However, in July 2014 (a month prior to the outbreak) the protodioscin concentrations of the simulated grazed samples were significantly higher when compared to the protodioscin concentrations in the plant material sampled by the total collection concentrations. The same occurred in August to November, 2014 (months corresponding to the outbreak) when occasion, eight lambs were hospitalized with signs of intoxication. Including clinical recurrences, there were 11 lamb hospitalizations in this period (Table 1).

The forage canopy presented different morphological characteristics that differed according to the time of the year. The samples obtained by the simulated grazing method were represented by growing leaf blades, belonging to the upper herbaceous stratum of the canopy, corresponding to the parts most selected by the lambs during grazing. The leaf blades obtained by the total collection method, after the morphological separation, were characterized by recently expanded or not, and senescence leaf blades.

In May, June and July of 2014 rainfall was respectively 118.00, 54.20, and 119.20mm. In May and June, the protodioscin concentrations on samples collected by either method were similar. In July, there was a significant difference between the protodioscin concentrations in the samples collected by the two methods. The protodioscin concentration in the samples obtained by simulated grazing remained constant in June and July. However, it was observed that in samples obtained by the total collection method, there was a variation of 0.75%-0.24% in the protodioscin concentrations (Fig.1).

In the months of August-November, 2014 rainfall was respectively 17.20, 65.80, 19.00 and 225mm. At this stage, the protodioscin concentrations of the samples obtained by simulated grazing were significantly higher than those in the samples obtained by total collection. From August to October 2014, the highest concentrations of protodioscin were observed in both collection methodologies (Fig.1).

As of November 2014, when there was an increase in rainfall, there was a drop in the protodioscin concentrations in samples obtained by both collection methods. In December 2014, January and February 2015, rainfall was respectively 364.00, 245.80 and 161.00mm and there was no significant difference between protodioscin concentrations in samples obtained by either collection method (Fig.1).

Table 1. Protodioscin concentrations (%) in *Brachiaria* spp. samples obtained by the simulated grazing (SG) and total collection (TC) methods

Stages	Month of collection*	Number of *Brachiaria*- poisoned sheep		Method	
		New cases (n = 8)	Relapses (n=12)	SG	TC
				Protodioscin (%)	
Suckling	May 2014			0.42	0.24
	June 2014			0.69	0.75
	July 2014**			0.68	0.24
Rearing	August2014**	5		1.61	1.14
	September 2014**	2	4	1.81	0.94
	October 2014**	1	1	1.27	0.89
	November 2014**		6	1.15	0.67
Finishing	December 2014			0.79	0.90
	January 2015			0.68	0.74
	February 2015		1	1,10	1.22
Average				1.020	0.777
Standard deviation				0.448	0.328

* Test: SG = TC *vs* SG ≠ TC is significant in 0.3447, nonsignificant by the test of Mann-Whitney (P>0.05); ** Test: SG = TC *vs* SG ≠ TC is significant in 0.0002, significant by the test of Mann-Whitney (P>0.05).

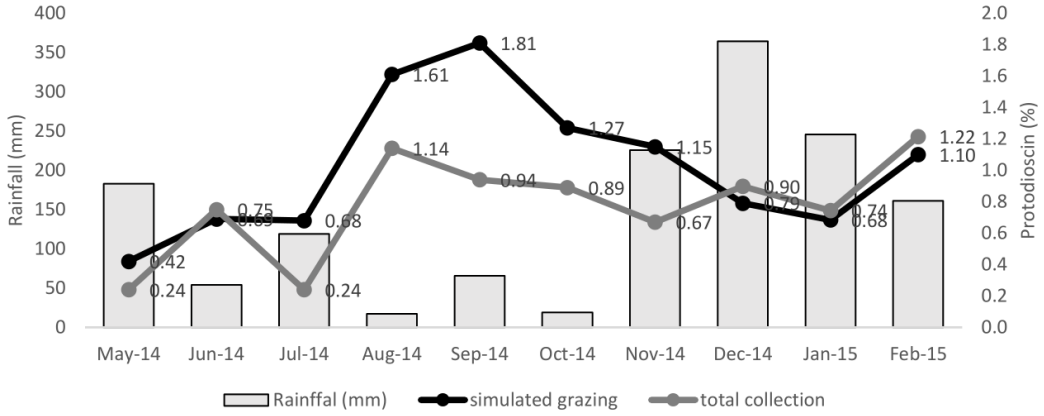


Fig.1. Protodioscin concentrations (% dry matter) in pastures of *Brachiaria* spp. from May 2014 to February, 2015. Data gathered through the analysis of samples obtained by the simulated grazing and the total collection methods.

DISCUSSION

When considering the whole experimental period, there was no significant difference between protodioscin the values obtained by the two methods of collection, demonstrating that both are effective for determining the concentrations of protodioscin in *Brachiaria* spp. However, from July to November 2014, the protodioscin concentrations values obtained by the simulated grazing method were higher than those obtained by the total collection concentrations. In July, one month before they experience an outbreak of *Brachiaria*, the lambs were still suckling and allocated to pastures of *B. brizantha*. The protodioscin concentration measured by the total collection method was 0.24%, whereas that measured by the simulated grazing method was 0.68%. Previous studies have reported outbreaks of *B. brizantha* poisoning in weaned lambs, where protodioscin concentrations ranged from 0.88% to 0.92% (Albernaz et al. 2010).

On August 2014 the lambs were weaned and transferred to pastures of *B. decumbens*. The highest protodioscin concentrations were observed from August to November 2014: protodioscin concentrations measured by the total collection method, ranged from 0.67% to 1.14%, while when measured simulated grazing methods were 1.15%-1.81%. An outbreak of *Brachiaria*-poisoning occurred during this same period, causing the hospitalization of eight lambs and clinical relapses of the toxicosis in 11 lambs. Previous studies have described outbreaks in less than one-year-old lambs, kept in pastures with mixed *Brachiaria* species or in pastures consisting exclusively of *B. decumbens*; such outbreaks were associated with protodioscin concentrations of 0.30%-2.56% (Mustafa et al. 2012, Porto et al. 2013). The protodioscin concentrations of the current study, measured by both methodologies, are within the concentrations range reported in previous studies which described outbreaks in sheep of the same category. However, those studies do not lend themselves to an adequate comparison, because they applied different forms of plant material collection and different intervals between collections (Mustafa et al. 2012, Porto et al. 2013). Furthermore, they sampled the entire aerial part of the plant (leaf blade, stem + sheath, and senescent material) at intervals of 7-15 days.

Although there is a great deal of information on the protodioscin concentrations and the different methods of plant collection, there were no previous studies comparing different methods for *Brachiaria* spp. collection for protodioscin levels determination, which at the same time associate these concentrations to the clinical manifestations of *Brachiaria* poisoning. Also, the feeding habits of sheep, which are more selective when compared to cattle, were not taken into account in previous studies (De Paula et al. 2009).

During the period of an outbreak of *Brachiaria* poisoning (August-November 2014) the samples of leaf blades acquired by the total collection method were formed by expanded or senescent leaf blades. On the other hand, the samples obtained by the simulated grazing consisted of growing or newly expanded leaf blades, belonging to the upper stratum of the canopy, corresponding to the parts most selected by the lambs. From these results, it is possible to demonstrate that the protodioscin concentrations in the growing leaf blades, which are more selected by the sheep, are significantly higher than the concentrations belonging to other parts, which tended to be less grazed. Thus, it can be stated that the collection by the methodology of simulated grazing allows a closer estimate of the concentrations of protodioscin responsible for the occurrence of clinical cases of intoxication. In other words, the occurrence of clinical cases and relapses concomitantly with the highest concentrations of protodioscin in growing leaf blades is related to grazing behavior of the lambs (De Paula et al. 2009, Faccin et al. 2014).

Samples obtained from both methodologies yield results with differences and similarities, which varied according to the period of the year. The highest protodioscin concentrations in samples from both methodologies were observed in the dry season, periods with lower rainfall (July-November 2014). From July to November 2014, there was a decrease in the production of new leaf blades and increase of leaf blades fibrous fraction. In the total collection methodology, there was a predominance of samples with senescent material which differed from that obtained by the selective grazing method, which had a predominance of growing or expanded leaf blades. In the dry season, plant moisture decreases and dry matter content increases consequently the protodioscin concentrations of increase in the growing leaflets commonly presented in samples obtained by the selective grazing method. As the samples obtained by total collection method consisting, almost in their entirety, of stems and senescent leaves, the

protodioscin concentration of the toxic principle decreased. As of November 2014, parallel with the increase in rainfall, there was an increase in growing leaf blades in the samples obtained by the total collection method.

During December 2014-2015, there was an increase in rainfall. An increment of forage mass and a higher prevalence of growing leaf blades were observed in the samples obtained by the total collection method, i.e., a sample similar to what the sheep selected in the selective grazing. The values of protodioscin were then approximately the same in samples gathered by both methods, because in both the presence of the same fraction prevailed and corresponded to new leaf blades not yet expanded. As these leaf blades have a high moisture content, the concentrations of protodioscin expressed as dry matter decreased because the fibrous fraction in which the toxic principle is associated was low.

Although the highest concentrations of protodioscin in the samples obtained by of simulated grazing coincided with the period of appearance of the clinical cases, it was not possible to determine the toxic protodioscin concentration for lambs due to the different degrees of disease observed in the poisoned sheep. In the present study, the cases occurred only in lambs originating from a sensitive herd, a progeny from ram and ewes showing clinical signs of intoxication in a given time (Pupin et al. 2016). The existence of varying degrees of sensitivity is related to genetic factors and the adaptation of sheep to the consumption of *Brachiaria* spp. (Faccin et al. 2014, Castro et al. 2018, De Melo et al. 2018).

CONCLUSION

Both methodologies used are adequate for determining the protodioscin concentration in *Brachiaria decumbens* in the pasture. However, specifically in periods of ongoing outbreaks of *Brachiaria* poisoning in sheep, the simulated grazing method is more efficient, as it more accurately depicts the amount of protodioscin ingested by the sheep.

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

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Bacterial identification, somatic cell count, antimicrobial profile and toxigenic *Staphylococcus* strains search from mastitic cow milk samples on small farms properties[1]

Ubirajara L. Lavor[2], Felipe F. Guimarães[2], Anelise Salina[2], Mateus S.R. Mioni[2] and Helio Langoni[2]*

ABSTRACT.- Lavor U.L., Guimarães F.F., Salina A., Mioni M.S.R. & Langoni H. 2019. **Bacterial identification, somatic cell count, antimicrobial profile and toxigenic *Staphylococcus* strains search from mastitic cow milk samples on small farms properties.** *Pesquisa Veterinária Brasileira 39(9):715-722*. Departamento de Higiene Veterinária e Saúde Pública, Universidade Estadual Paulista, Rua Prof. Dr. Walter Maurício Correa s/n, Campus Botucatu, Botucatu, SP 18618-681, Brazil. E-mail: helio.langoni@unesp.br

Bovine mastitis has a negative impact on milk production and can pose risks to public health. The present study aimed to evaluate the quality of bovine milk from small farms in the Botucatu/SP region. Somatic cell counts (SCC), identification of pathogens involved in mastitis, and sensitivity antimicrobial profile of staphylococci isolated were performed. The presence of enterotoxin encoding genes in isolates of staphylococci obtained from milk was investigated. Milk samples from individual mammary quarters of cows were submitted to the California mastitis test (CMT) and SCC. Of the 239 dairy cows from 21 dairy herds evaluated (mean = 11.4 animals/property), two cows (0.8%) presented clinical mastitis and 86 (35.9%) subclinical mastitis. Bacterial culture was performed in 177 quarter milk samples. Staphylococci were identified in 55 (31.1%), corynebacteria in 45 (25.4%), streptococci in 25 (14.1%) and coliforms in four (2.3%) milk samples. Average SCC from culture-positive samples was $1598x10^3$ cells/mL, in case of staphylococci was $1362x10^3$ cells/ml, streptococci was $2857x10^3$ cells/mL, corynebacteria was $976x10^3$ cells/mL and in the cases of coliforms $1161x10^3$ cells/mL were obtained. Staphylococci showed a high sensitivity (>95%) to cephalothin, cotrimoxazole, enrofloxacin, and gentamicin, with a 41.2% resistance to penicillin and 11.8% to oxacillin. Both coagulase positive (CPS) and negative staphylococci (CNS) carried genes encoding enterotoxins in 21.6% of the first group and 41.9% in the second. The *sea* gene was the most detected 45.8% (n=24) between them, followed by *seb* with 29.2% and *sec* with 25.0%. The *sed* gene was not identified. We highlight the potential risk to public health in the possibility of strains of *Staphylococcus* spp. enterotoxin-producing genes that can cause staphylococcal food poisoning.

INDEX TERMS: Bacterial identification, somatic cell count, antimicrobial profile, toxigenic, *Staphylococcus,* mastitis, cow, milk, farms, enterotoxins, public health, gene, *sea*, *seb*, *sec*.

RESUMO.- [**Identificação bacteriana, contagem de células somáticas, perfil antimicrobiano e pesquisa de linhagens toxigênicas de *Staphylococcus* em amostras de leite bovino de pequenas propriedades rurais.**] A mastite bovina impacta negativamente a produção leiteira e pode acarretar riscos à saúde pública. O presente estudo teve como objetivo a avaliação da qualidade do leite bovino proveniente de pequenas propriedades na região de Botucatu/SP. Foi realizada a contagem de células somáticas (CCS), identificação dos patógenos envolvidos nas mastites, e realizado o perfil de sensibilidade aos antimicrobianos dos estafilococos isolados. Pesquisou-se a presença de genes codificadores de enterotoxinas em isolados de estafilococos obtidos a partir do leite mastítico.

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[2] Departamento de Higiene Veterinária e Saúde Pública, Universidade Estadual Paulista (Unesp), Rua Prof. Dr. Walter Maurício Correa s/n, Campus Botucatu, Botucatu, SP 18618-681, Brazil. *Corresponding author: helio.langoni@unesp.br

Amostras de leite de quartos mamários individuais de vacas foram submetidas ao "California mastitis test" (CMT) e à CCS. Das 239 vacas em lactação provenientes de 21 rebanhos leiteiros avaliados (média = 11,4 animais/propriedade), dois (0,8%) animais apresentaram mastite clínica e, 86 (35,9%) mastite subclínica. 177 amostras de leite foram cultivadas em ágar sangue bovino 5% e ágar MacConckey e obteve-se 55 (31,1%) *Staphylococcus* spp., 25 (14,1%) *Streptococcus* spp., 45 (25,4%) *Corynebacterium* spp. e quatro (2,3%) coliformes. A média da CCS das amostras procedentes de todos os quartos mamários infectados avaliados foi de 1598x10^3 células/mL, enquanto que nos casos que foram isolados *Staphylococcus* spp. foi de 1362x10^3 células/mL, *Streptococcus* spp. 2857x10^3 células/mL, *Corynebacterium* spp. de 976x10^3 células/mL e nos casos de coliformes 1161x10^3 células/mL. Os estafilococos revelaram grande sensibilidade (>95%) à cefalotina, cotrimoxazol, enrofloxacina e gentamicina, com resistência de 41,2% à penicilina e 11,8% à oxacilina. Tanto estafilococos coagulase positivos (ECP) como negativos (ECN) revelaram genes codificadores de enterotoxinas em 21,6% do primeiro grupo e 41,9% no segundo. O gene *sea* foi o mais detectado 45,8% (n=24), seguido pelo *seb* com 29,2% e *sec* com 25,0%. O gene codificador da *sed* não foi identificado. Frente aos resultados, destaca-se o risco potencial à saúde pública pela possibilidade de veiculação de linhagens de *Staphylococcus* spp. carreadores de genes produtores de enterotoxinas, podendo ocasionar toxi-infecções alimentares.

TERMOS DE INDEXAÇÃO: Identificação bacteriana, contagem de células somáticas, *Staphylococcus,* leite, bovinos, propriedades rurais, mastite, enterotoxinas, saúde pública, genes, *sea*, *seb*, *sec*.

INTRODUCTION

Approximately 85% of the 1.3 million dairy farmers in Brazil can be classified as small producers. Low technification, poor management, poor sanitary control and inadequate hygiene conditions decrease the quality of the raw milk produced, posing a risk to consumers of the transmission of micro-organisms that cause zoonoses or possible toxi-infections.

Mastitis, the most prevalent disease in dairy cattle, affects the mammary gland leading to physicochemical and bacteriological changes in milk. Infectious causes are the most significant, both economically and in the public health interest. SCC is considered a good indicator of mammary gland health because it assesses the number of inflammatory cells present in milk. At counts above 200,000 cells/mL, fourth breasts are considered positive for mastitis (Dohoo & Leslie 1991).

Determining the etiology of mastitis enables the adoption of appropriate antimicrobial treatment. The differentiation of the contagious or environmental factors of the infection, the adoption of disease prevention and control measures are distinct for each group of microorganisms (Langoni et al. 1998, Radostits et al. 2007).

Despite the recent regulation of the dairy chain has led to transformations in the sector, such as the way of collection, with the bulk collection and the end of receiving uncooled milk, beginning of payment policies for quality, incorporation of technologies in the field and development of new products and industrial processes, the difficulties faced by small farmers in adapting their production to the new quality parameters established are still big (Carvalho 2010).

Due to the impossibility of meeting the pressures imposed by the market or lack of motivation due to the little consolidation of public policies in the dairy sector, many producers end up joining the informal market. Risking illegality, this producer can add to his production part of the income that would be appropriated by the intermediaries. (Silva & Tsukamoto 2001). As a result, this informally traded milk exposes consumers to greater risks, with serious public health implications.

Hygienic obtaining of milk, in addition to directly impacting product quality, increases the productivity of the herd by decreasing the prevalence of intramammary infections, resulting in direct and indirect economic gains for farmers (Langoni et al. 2011).

Staphylococcus is one of the main agents involved in the prevalence and persistence of mastitis in the dairy herd. (Fagundes & Oliveira 2004, Piepers et al. 2007). *Staphylococcus aureus* stands out for its difficult control due to its high resistance to the antimicrobial treatments used and the various virulence factors, causing long-lasting infections with low cure rates (Silva et al. 2014, Guimarães et al. 2017). Another important feature is the ability to produce enterotoxins, which can lead to poisoning by ingestion of thermostable toxins (Pinchuk et al. 2010) produced and released during their multiplication in food (Soriano et al. 2002).

Staphylococcal enterotoxins (SE) are a group of low molecular weight single chain proteins produced during all stages of bacterial multiplication, but especially during the half and end of the exponential phase (Soriano et al. 2002). Classic enterotoxins (A, B, C, and D) are the most common in bovine mastitis milk isolates. Their production is not restricted to *Staphylococcus aureus* species, but also to other staphylococci species such as coagulase negative staphylococci (CNS) (Radostits et al. 2007, Freitas Guimarães et al. 2013). The importance of the genus and its toxins is well elucidated in the literature, however, there is little information on the occurrence of genes that encode these toxins in the milk of animals from small dairy herds.

In order to reduce intramammary infections, it is essential to develop orientation and support activities for producers, such as extension activities, lectures and training directed to them. In order to achieve this reduction, it is recommended to improve the diagnostic techniques of clinical and subclinical mastitis, laboratory identification of the infectious agents involved, appropriate antimicrobial treatment of the affected quarters, cleaning and disinfection of the teats (pre and post-dipping) as well as the environment, in addition to the provision of good quality water to ensure the proper development of the inherent production activities.

This study aimed to investigate the aerobic microbiota of subclinical mastitis cases observed in small dairy properties, the relationship between the isolated species and the milk SCC of the respective breast quarters positive for the CMT test. It also aimed to identify the etiology of mastitis, as well as the potential risks to public health by detecting enterotoxin coding genes in strains of *Staphylococcus* spp. and established the antimicrobial sensitivity profile of staphylococci isolates obtained.

MATERIALS AND METHODS

Microbiological analyses, somatic cell count and molecular studies were performed at the Mastitis Research Center (NUPEMAS) of the Department of Veterinary Hygiene and Public Health, School of Veterinary Medicine and Animal Science, Unesp, Botucatu Campus/SP.

Dairy properties. The study was conducted in dairy herds whose daily production was less than 100 liters of milk. There were 21 distinct herds located in the rural area of Botucatu/SP. The number of lactating cows per herd ranged from 3 to 20, predominantly crossbred animals.

Mastitis diagnosis and sample collection. We screened all lactating cows from each herd. The diagnosis of clinical mastitis was based on clinical signs of breast inflammation (pain, flushing, heat and swelling) and/or changes in milk macroscopy (lumps, pus, blood or filaments) by proof of dark-bottomed cup or Tamis (Radostits et al. 2007). Subclinical mastitis was diagnosed with the aid of CMT (Schalm & Noorlander 1957). Tamis-positive milk samples with a CMT positivity score were collected aseptically, according to pre-established procedures (NMC 1999). Until microbiological examinations are performed immediately upon arrival at the laboratory, the samples were kept refrigerated (4 to 8°C) in isothermal boxes with recyclable ice.

Microbiological culture. The microbiological culture was performed according to standard procedures (NMC 1999). Initially, 10 microliters of each milk sample were grown in 5% bovine blood agar and MacConkey agar media, incubating at 37°C, observing microbial development every 24 hours for three days. The isolated colonies were picked in BHI (brain heart infusion) broth, studied morphologically for phenotypic characteristics (pigmentation and hemolysis), as well as morphologically by the Gram technique, and classified according to their ability to synthesize the coagulase enzyme in order to differentiate coagulase positive (CPS) from negative staphylococci (CNS). We used other biochemical tests to complement the classification of the other species and subspecies of CNS, such as the fermentation of sugars: mannitol, maltose, trehalose, xylose, arabinose, sucrose, lactose, xylitol, ribose and fructose. We observed urease and/or ornithine decarboxylase production, nitrate reduction and susceptibility to novobiocin (Cunha et al. 2007). For identification of enterobacteria, colonies isolated from MacConkey were subjected to biochemical tests of EPM, MILi and Simmons Citrate, where the production of gas, glucose, enzyme L-tryptophan deaminase (LTD), H2S, urea, motility, indole and lysine were evaluated (Trabulsi et al. 1999). Colonies suggestive of streptococci were submitted to catalase test, esculin hydrolysis and CAMP test, aiming to differentiate *Streptococcus agalactiae* from other species of mastitis-causing streptococci (Quinn et al. 2005).

Animals' SCC. In parallel to the above procedure, they were collected in a plastic bottle containing bronopol cell preservative (Bertrand 1996), Tamis or CMT-positive breast milk samples. SCC was performed on Somacount 300® equipment (Bentley, UK) by flow cytometry.

Microbial Sensitivity Profile. *Staphylococcus* spp. isolates were tested for microbial sensitivity by the disk diffusion method recommended by Bauer et al. (1966), on plates containing Mueller Hinton agar, arranged with the following antimicrobials: neomycin (30μg), gentamicin (10μg), penicillin (10UI), oxacillin (10μg), cephalothin (30μg), enrofloxacin (5μg) and cotrimoxazole (25μg). Interpretation of inhibition halos followed the Clinical and Laboratory Standards Institute (CLSI 2005) reference.

DNA extraction. *Staphylococcus* spp. were cultured in BHI broth, concentrated by centrifugation and DNA extracted using the Illustra Blood Genomic Prep Mini Spin Kit (GE Healthcare), as recommended by the manufacturer, after initial cell digestion with lysozyme (10mg/mL) and Proteinase K (20mg/mL).

Detection of enterotoxin coding genes. We used specific primers for the EEA (*sea*), EEB (*seb*), EEC (*sec*) and EED (*sed*) enterotoxin coding genes, according to Johnson et al. (1991). Reactions were performed in 0.2mL microtubes in a total volume of 25μL, with 10pmol of each primer, 1.0U Taq Platinum DNA polymerase (Invitrogen®), 200μM triphosphate deoxyribonucleotides, 1X PCR buffer, 0.75mM of MgCl2 and 3μL of sample. In all reactions negative controls were used with the replacement of nucleic acid by water. Incubation was performed on the Mastercycler gradient thermocycler (Eppendorf®).

Visualization of amplified products. Agarose gels were prepared in 2.0% concentration in 1X TBE buffer, stained with 1.0μL/mL SYBR Safe DNA gel stain (Invitrogen®). Products size were compared to the 100bp standard and subsequently photographed under UV transillumination.

Statistical analysis. The results were analyzed by general descriptive statistics, using the Windows Excel® program. Mixed linear models were used to compare the mean milk SCC between mammary glands infected with different pathogens. We used logistic regression models or Chi-square tests to test the association between *Staphylococcus* spp. and the antimicrobial sensitivity profile.

RESULTS AND DISCUSSION

Mastitis etiology

The results obtained for isolated species in the microbiological culture of 177 lactating cow's milk samples showed the occurrence of important mastitis pathogens and are presented in Table 1. The predominance of the genus *Staphylococcus* agrees with other prevalence studies conducted in both Brazilian dairy herds and in dairy farms in other countries (Nader Filho et al. 2007, Bolaños et al. 2014).

Corynebacterium spp. also plays an important role in the etiology of mastitis. *C. bovis* is responsible for significant reduction in milk production of infected fourth breasts. When evaluating the presence of *C. bovis* in dairy herds, Domingues et al. (1998) observed a decrease in milk production of up to 27.6%, while Zafalon et al. (1999) reported a 30.9% drop, in addition to observing a significant increase in milk SCC in animals with these bacteria.

Of the 31 isolates classified as *Streptococcus*, 67.7% (n=21) of these were classified as *S. agalactiae,* 25.8% (n=8) as *S. dysgalactiae* and 6.5% (n=2) as *S. bovis*. The main contagious agent of the genus Streptococcus and frequently isolated in the etiology of bovine mastitis is *S. agalactiae*. It is a mandatory and highly contagious intramammary microorganism (González et al. 1986) and can be efficiently eliminated with intramammary treatments during the lactation period.

Table 1. Bacterial isolation in milk samples from cows with clinical and subclinical mastitis in small dairy farms

Bacterial genus	Total (N)	Total %
Staphylococcus spp.	55	31.1
Corynebacterium spp.	45	25.4
No growth/contaminated*	34	19.2
Streptococcus spp.	25	14.1
Corynebacterium spp./*Staphylococcus* spp.	8	4.5
Staphylococcus spp./*Streptococcus* spp.**	5	2.8
Coliforms	4	2.3
Corynebacterium spp./*Streptococcus* spp.**	1	0.6
TOTAL	177	100

*No growth/contaminated = no bacterial growth samples or growth of three or more different microorganisms (NMC 1999).

Coliforms are important indicators of the hygienic and sanitary quality of food and frequently participate in breast infections. In the present study, only 2.3% (n=4) of the samples were found. Freitas Guimarães et al. (2013) and Ribeiro et al. (2009) also reported prevalence of Enterobacteriaceae-related mastitis in dairy cows.

The high prevalence of contagious microorganisms of the genera *Staphylococcus, Streptococcus* and *Corynebacterium* is due to the habitat of this group consisting of the mucosa and skin of the animals, indicating problems related to the operational hygiene of milking and possible chronicity of breast infection.

Regarding the biochemical characterization of staphylococci (Table 1), the main isolated species was *S. aureus* (n=21). We also observed the participation of CNS as important pathogens in bovine mastitis, as reported by Piepers et al. (2007).

Staphylococcus aureus and *S. intermedius* are causative agents of bovine mastitis, the latter being less frequently isolated in milk samples (Roberson et al. 1996, Oliveira et al. 2011, Freitas Guimarães et al. 2013). These species may be responsible for the occurrence of food poisoning outbreaks when associated with the presence of enterotoxin coding genes (Radostits et al. 2007, De Freitas Guimarães et al. 2013, Rall et al. 2014). *S. hyicus* is appointed as responsible for the production of exotoxin from scalded skin syndrome in pigs (Tanabe et al. 1996). Chenier & Lallier (2012) also attributed to this species some infections in laying hens. In this sense, the presence of other animals, such as swine and poultry on small dairy farms, may have contributed to the participation of this genus in the observed mastitis.

Regarding the species of CNS, the present study agreed with the scientific literature that indicated frequent participation of *S. hominis* and, to a lower frequency of *S. sciuri, S. capitis* (Radostits et al. 2007). *S. epidermidis* is more prevalent and persistent in human skin and mucous membranes. It can be inferred that its prevalence may be directly related to precariousness in the milking procedures in the visited properties, being necessary the confirmation from the bacteriological swab examination of the hands of the milkers.

Somatic cell count

Milk SCC reflects the intensity of the inflammatory response to breast tissue infection, and the values obtained in the present study are presented in Table 2. Defense cells, mainly neutrophils, migrate into the udder to eliminate the pathogen. In addition to the genetic characteristics and immunological capacity of animals, SCC is directly influenced by lactation period, month and season, order of birth, among others. However, the specific effect of pathogens and the state of breast infection are the main factors responsible for their variations.

The major pathogens (*S. aureus*, *S. agalactiae* and coliforms) cause mastitis, which results in large variations in milk composition and SCC, while secondary pathogens (CNS and *C. bovis*) cause moderate inflammatory process (Harmon 1994). The results obtained in this study corroborate those found by Souza et al. (2009) in which *S. agalactiae* was responsible for the largest increase in dairy cow SCC.

Comparison between the SCC values obtained within the *Staphylococcus* genus for the CPS and CNS groups revealed that the mean value of the first group ($1632x10^3$ cells/mL) was significantly higher than the average observed in cases of CNS ($1060x10^3$ cells/mL). However, the comparison of the observed maximum values, $7139x10^3$ CS/mL for CPS and $7357x10^3$ CS/mL for CNS, demonstrates the ability of the latter to elicit high intensity inflammatory processes.

Antimicrobial resistance

The genus *Staphylococcus* showed high sensitivity to most antimicrobials. Cephalothin, cotrimoxazole, enrofloxacin and gentamicin were the most effective antimicrobials (>95.0%). However, we noted worrying indexes of intermediate sensitivity or complete resistance, compared to some drugs, especially penicillin (41.2%), oxacillin (11.8%) and neomycin (5.8%) (Table 3).

Bacterial resistance is related to the existence of genes capable of encoding different biochemical mechanisms that give the microorganism the ability to resist the action of various drugs. It is the result of selective pressure on pathogens that occurs through the use or not of medicines (Tavares 2000).

Table 2. CPS and CNS strains isolated from milk of cows with mastitis in small dairy farms

Group	Species	N	% in the group	% in the genus	% in the isolates
	Staphylococcus spp.	9	24.3	13.2	6.3
CPS	*S. aureus*	21	56.8	30.9	14.7
	*S. hyicus**	4	10.8	5.9	2.8
	S. intermedius	2	5.4	2.9	1.4
	S. schleiferi	1	2.7	1.5	0.7
	Subtotal	37	100.0	54.4	25.9
	Staphylococcus spp.	8	25.8	11.8	5.6
	S. xylosus	9	29.0	13.2	6.3
CNS	*S. epidermidis*	6	19.4	8.8	4.2
	S. chromogenes	5	16.1	7.4	3.5
	S. warneri	2	6.5	2.9	1.4
	S. simulans	1	3.2	1.5	0.7
	Subtotal	31	100.0	45.6	21.7
	TOTAL	68	-	100.0	47.6

**S. hyicus* isolates obtained in this study were grouped together with other CPS for positive coagulase test results.

In addition to being a serious animal and public health problem, antimicrobial resistance results in direct financial losses to dairy farmers due to drug costs and milk disposal during treatment. They also lose dairy products and end consumers. When the recommended grace periods for antimastitics are not respected, the quality and safety of dairy products are compromised.

In a large study conducted in the state of Minas Gerais, Costa et al. (2013) evaluated the antimicrobial profile of 352 isolates of *Staphylococcus aureus* obtained from 35 different dairy properties. Resistance percentages obtained for cephalothin (0.28%), gentamicin (1.69%), neomycin (3.35%), enrofloxacin (<1.0%) and penicillin (34.1%) were very similar to our results.

Regarding penicillin, the staphylococcal strains isolated in the present study showed a sensitivity percentage of 58.8% (n=40) of isolates sensitive to this antimicrobial, 39,7% (n=27) of the isolates resulted in intermediate antimicrobial sensitivity and 1.5% (n=1) was resistant. In small dairy herds, low technification and probable deficiencies in therapeutic protocols could result in higher levels of microorganism resistance to penicillin, widespread antimicrobial agent in the treatment of mastitis in dairy herds (Vintov et al. 2003, Rabello et al. 2005). Contrary to what would be expected in isolates obtained from this type of herd, only one isolate resistant to the drug in question was observed.

Regarding oxacillin, the total 11.8% of intermediate sensitivity and resistance observed is like those obtained by Siqueira (2011) in bacteria of this genus isolated from samples of organic milk in the Botucatu region, in which 88% were sensitive to the drug.

The indiscriminate use of penicillin reported by the producers of this study may have contributed to the selective pressure of strains resistant to this antimicrobial, as compared the profiles obtained for groups of CPS and CNS. In the first, 48.6% of the isolates were considered sensitive and 51.4% were resistant to the drug, while the microorganisms of the CNS group presented 71.0% sensitivity and 25.8% resistance. The fact that CPS induces a more intense inflammatory response in cases of mastitis tends to use antimastitic treatments more frequently than CNS, and may lead to selective pressure and consequent resistance of microorganisms to the drug.

Enterotoxins

Of the 68 samples subjected to enterotoxin-encoding gene screening 30.9% (n=21) were positive for at least one type of classical enterotoxin encoding gene (*sea*, *seb*, *sec* or *sed*). The frequency of the CPS and CNS species, and the association with the genes are presented in Table 4.

Of the 37 CPS samples submitted to PCR, 21.6% (n=8) were positive for at least one of the classic enterotoxin coding genes and of the 31 CNS samples, the positivity was 41.9% (n=13). De Freitas Guimarães et al. (2013) obtained a significantly higher positivity of the CNS in relation to the CNS in the research of the same genes.

Considering only samples positive for the detection of enterotoxin coding genes, it was verified that the gene *sea* was the most prevalent with 45.8%, followed by *seb* with 29.2% and *sec* with 25% frequency. The *sed* gene was not detected in any of the samples. These results partially agree with those found in the scientific literature. Zoli et al. (2002) pointed out that sed-expressed enterotoxin is the second main type involved in food outbreaks, with bone poisoning being more frequent. Pinchuk et al. (2010) also associated food poisoning with *sed* detection.

The study by Pimentel et al. (2002) in composite milk samples revealed the production of some type of enterotoxin in 24.6% of CPS samples and 41.3% of CNS samples. The authors concluded that coagulase negative species produce enterotoxins more frequently, highlighting the lack of updated Brazilian

Table 3. Average SCC of milk from fourth breasts of subclinical mastitis cows according to bacterial genus

SCCx10^3 (CS/mL)	STA	STR	COR	COLIF.	STA/STR	COR/STA	COR/STR
Average	1362	2857	976	1161	3707	1904	-
Mean	579	1456	501	465	4501	934	608
Maximum	7357	9999	6554	3369	9999	4655	-
Minimum	124	175	42	319	375	539	-
N	55	25	45	4	5	8	1
%	38.5	17.5	31.5	2.8	3.5	5.6	0.7

STA = *Staphylococcus* spp., SRT = *Streptcoccus* spp., COR = *Corynebacterium* spp., COLIF. = coliform groups, STA/STR = genus *Staphylococcus* spp. and *Streptococcus* spp., COR/STA = genus *Corynebacterium* spp. and *Staphylococcus* spp., COR/STR = genus *Corynebacterium* spp. and *Streptococcus* spp.

Table 4. Sensitivity profile of *Staphyloccocus* spp. against different antimicrobials

Antimicrobial	*Staphylococcus* spp.					
	Sensitive		Intermediate		Resistant	
	N	%	N	%	N	%
Cephalothin (30µg)	67	98.5	1	1.5	0	0.0
Cotrimoxazole (25µg)	66	97.1	1	1.5	1	1.5
Enrofloxacin (µg)	65	95.6	1	1.5	2	2.9
Gentamycin (10µg)	66	97.1	2	2.9	0	0.0
Neomycin (30µg)	64	94.1	2	2.9	2	2.9
Oxacilin (10µg)	60	88.2	5	7.4	3	4.4
Penicilin (10UI)	40	58.8	27	39.7	1	1.5

Table 5. Frequency of enterotoxin coding genes in CPS and CNS isolated from milk samples from small dairy farms

CPS	Samples results		Genes			
	Negatives	Positives	*sea*	*seb*	*sec*	*sed*
Staphylococcus spp.	8	1	1	-	-	-
S. aureus	17	4	3	-	2	-
S. hyicus	2	2	1	1	-	-
S. schleiferi	-	1	-	-	1	-
S. intermedius	2	-	-	-	-	-
Subtotal	29	8	5	1	3	0
CNS	Negatives	Positives	*sea*	*seb*	*sec*	*sed*
Staphylococcus spp.	4	4	2	2	1	-
S. chromogenes	2	3	-	2	1	-
S. xylosus	6	3	1	2	-	-
S. epidermidis	4	2	2	-	1	-
S. warneri	1	1	1	-	-	-
S. simulans	1	-	-	-	-	-
Subtotal	18	13	6	6	3	0

legislation (RDC no. 12/2001 of the National Health Surveillance Agency, Anvisa) that advocates for only CPS counting for risk assessment of enterotoxin production (Brasil 2001).

Nevertheless, as reported by Faccioli (2010), a high prevalence of enterotoxin coding genes does not necessarily correspond to high enterotoxin expression. Carmo et al. (2009) evidenced the presence of natural mechanisms capable of inhibiting enterotoxin production in milk contaminated with enterotoxigenic *S. aureus* strains. Factors such as bacterial growth conditions, presence of glucose, pH of the medium and even regulatory systems of the microorganism itself interfere with gene expression (Cunha et al. 2007).

The results in Table 5 showed that in both CPS and CNS strains, there are isolates that have genes encoding two types of staphylococcal enterotoxins. In *S. aureus* strains, the presence of two enterotoxin coding genes, *sea* and *sec*. In the CNS strains, *S. epidermidis* revealed the *sea* gene in association with *sec*. Another isolate from *Staphylococcus* spp. presented the *seb* and *sec* genes, reinforcing that it is possible that certain staph strains express concomitantly more than one enterotoxin-producing gene.

We showed the importance of milk in the circulation of staphylococcal strains with enterotoxigenic potential that may, under favorable conditions, lead to food poisoning. In the context of family farming, this potential risk is even more worrisome because milk and dairy products are ingested by producers and their families, in addition to the clandestine trade in milk.

CONCLUSIONS

The results of the present study revealed that there is great variability in the etiology of mastitis in dairy herds of small rural properties.

The high frequency of isolation of contagious species of the *Staphylococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. in the milk of the animals evidences the greater importance of contagious mastitis in the herds evaluated. SCC showed significantly different values according to the pathogen responsible for inflammation of the mammary gland.

In the antimicrobial sensitivity profile, the CPS presented higher resistance when compared to the CNS. The presence of enterotoxin coding genes A, B and C in *Staphylococcus* spp. demonstrated the potential risks to public health due to the possibility of food poisoning through the consumption of raw milk from this type of herd.

The multiple etiology of mastitis observed in the small dairy farms analyzed in the study reinforces the importance of microbiological evaluation before the adoption of milk quality control and monitoring programs. Therefore, we emphasize the importance of the implementation of indirect diagnostic tests, such as CMT or SCC, for the detection of subclinical mastitis for subsequent microbiological examination and success in control programs.

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

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Leukotriene B4 in equine asthma syndrome: what do we know so far?[1]

Juliana N.P. Otaka[2]*, Luis F.A. Toledo[2], Katia M. Silva[2], Andreza A. Silva[3], Laís Agra[4], Bruno L. Diaz[4] and Daniel A.B. Lessa[2]

ABSTRACT.- Otaka J.N.P., Toledo L.F.A., Silva K.M., Silva A.A., Agra L., Diaz B.L. & Lessa D.A.B. 2019. **Leukotriene B4 in equine asthma syndrome: what do we know so far?** *Pesquisa Veterinária Brasileira 39(9):723-727.* Universidade Federal Fluminense, Rua Vital Brazil Filho s/n, Niterói, RJ 24230340, Brazil. E-mail: ju.nabuco@gmail.com

The term "equine asthma syndrome" (EAS) was recently proposed due to the resemblance of the equine disease to human asthma. Leukotrienes cause constriction of the bronchi, especially in the lower airways and increase mucus secretion in the respiratory system. Leukotriene B4 (LTB4) has been discovered as a strong chemotactic factor, which plays a role in neutrophil migration. The immunologic background of EAS remains not fully elucidated despite many studies on the pathogenesis. This study aimed to evaluate the LTB4 concentration in the bronchoalveolar lavage fluid (BALF) of horses with and without pulmonary inflammatory disease. Thirty-five mixed breed horses were studied and LTB4 was determined by using specific ELISA Kit. The horses were grouped by 2 different criteria for statistical analysis of data: 1) according to the values for BALF citology and 2) according to the detection of LTB4 in BALF. There was significant difference of effect of age on the LTB4 detection in equine BALF. Younger animals were the majority where it was possible to detect LTB4 values in LBA. In conclusion, there was an effect of age on the detection of LTB4 in equine BALF, where LTB4 levels were more easily detected in younger animals than older animals and the results of this study raise the possibility of considering future studies with the objective of establishing the real role and the best moment to detect LTB4 in BALF of the equine asthma syndrome.

INDEX TERMS: Leukotriene B4, equine, asthma syndrome, BALF, horse, IAD, LTB4, RAO.

RESUMO.- [**Leucotrieno B4 na síndrome asmática equina: o que sabemos até o momento?**] Recentemente, o termo "síndrome da asma equina" (SAE) foi proposto devido à semelhança da doença equina à asma humana. Os leucotrienos causam constrição dos brônquios, especialmente nas vias aéreas posteriores e aumentam a secreção de muco no sistema respiratório. O leucotrieno B4 (LTB4) foi descoberto como um forte fator quimiotático, que desempenha um papel na migração de neutrófilos. O fundo imunológico do SAE permanece não completamente elucidado apesar de muitos estudos sobre a patogênese. Este estudo teve como objetivo avaliar a concentração de LTB4 no lavado broncoalveolar (LBA) de equinos com e sem doença inflamatória pulmonar. Trinta e cinco cavalos de raças mistas foram estudados e o LTB4 foi determinado usando o kit ELISA específico. Os animais foram agrupados por dois critérios diferentes para análise estatística dos dados: 1) de acordo com os valores para citologia do LBA e 2) de acordo com a detecção do LTB4 no LBA. Houve diferença significativa do efeito da idade na detecção do LTB4 no LBA equino. Os animais mais jovens foram a maioria onde foi possível detectar os valores de LTB4 no LBA. Em conclusão, houve um efeito da idade na detecção de LTB4 em LBA equino, onde os níveis de LTB4 foram mais facilmente detectados em animais jovens do que em animais mais velhos e foi possível detectar a concentração de LTB4 no LBA equino e os resultados deste estudo levantam a possibilidade de considerar futuros estudos com o objetivo de estabelecer o real papel e o melhor momento para detectar LTB4 no LBA da síndrome asmática equina.

TERMOS DE INDEXAÇÃO: Leucotrieno B4, síndrome asmática equina, equinos, cavalos, LBA, LTB4.

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[2] Departamento de Patologia Veterinária e Clínica, Faculdade de Veterinária, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho s/n, Niterói, RJ 24230340, Brazil. *Corresponding author: ju.nabuco@gmail.com

[3] Departamento de Clínica e Cirurgia, Faculdade de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), BR-465 Km 7, Seropédica, RJ 23890-000, Brazil.

[4] Laboratório de Inflamação, Instituto de Biofísica Carlos Chagas, Universidade Federal do Rio de Janeiro (UFRJ), Av. Carlos Chagas Filho 373, CCS, Bloco C, sala C1-024, Ilha do Fundão, Rio de Janeiro, RJ 21941-902, Brazil.

INTRODUCTION

Respiratory tract diseases stands out in equine medicine due to their high prevalence and impact on animal health and economic losses to the equine industry (Michelotto et al. 2013).

According to Barton & Gehlen (2016), disorders of the respiratory system are the most frequently diagnosed conditions in sport horses evaluated for poor performance, particularly in the lower airways such as equine recurrent airway obstruction (RAO) and inflammatory airway disease (IAD).

Recently, Couëtil et al. (2016) proposed the term "equine asthma syndrome" (EAS) due to the resemblance of the equine disease to human asthma. This term encompasses IAD, classified as mild to moderate asthma, and RAO, classified as severe asthma (Pirie 2017). IAD is a syndrome defined as a nonseptic lower airway condition affecting horses of any age, which usually exhibit subtle clinical signs at rest. The cytological profile of horses with IAD might reveal an increased total nucleated cells count and a very heterogeneous cell population with mild neutrophilia or, alternatively, increased eosinophil counts in equine BALF, while RAO is associated with severe neutrophilic airway inflammation (Richard et al. 2014).

Human asthma has been commonly described as an eosinophilic disease, however it is lately more and more recognized that neutrophilic inflammation may be present in human asthma of all severities as well, but in particular in severe asthmatic patients and during acute disease exacerbation (Nakagome et al. 2012). On the other hand, eosinophils, metachromatic cells, or neutrophils characterize the subforms of mild-to-moderate equine asthma or IAD (Couëtil et al. 2016).

Since the use of fiber-optic endoscopy was first described in horses, cytological and microbiological evaluation of BALF have become the pillars in the diagnosis of respiratory disease alongside clinical and functional examinations (Barton & Gehlen 2016). Although severely asthmatic horses often show easily visible clinical signs of disease, difficulties may arise in clinical practice due to the fact that patients may be presented in remission. Mild-to-moderate equine asthma tends to occur subclinically as well (Pirie 2017). In these cases, commonly used examination techniques may be insufficient for diagnosis and evaluation of treatment success.

Leukotrienes represent a biologically active group of low molecular weight lipid mediators (i.e., eicosanoids) formed from arachidonic acid. 5-Lipoxygenase (5-LO) is a primary component to this process, initiating the oxidation of arachidonic acid and the subsequent production of intermediate metabolites (e.g., 5-oxe-eicosatetraenoic acid), promoting proinflammatory cell recruitment as well as the eventual formation of the prominent leukotriene subtypes (Ochkur et al. 2013).

Martin et al. (1989) reported that unlike other products of this pathway as leukotrienes C4, D4, and E4, which are potent bronchoconstrictors in humans and animals, the major effects of LTB4 are directed at phagocytes. When studied in vitro, LTB4 promotes chemotaxis of polymorphonuclears (PMN), monocytes, and fibroblasts.

According to Ochkur et al. (2013), the production of leukotrienes is linked with a variety of leukocytes that are both resident and recruited in response to allergen provocation, including neutrophils, eosinophils, mast cells, macrophages, and basophils.

Neutrophils are the most abundant leukocytes in the bloodstream and the first cells to be recruited to a site of inflammation, where primary chemotactic ones are produced, such as formyl-peptides released from bacteria or necrotic cells and complement fragments. Locally, primary chemoattractant gradients recruit more neutrophils into the nucleus of inflammation. After they have reached the site of inflammation, these neutrophils secrete secondary chemoattractants and recruit additional leukocytes, further amplifying the inflammation process. These proinflammatory mediators activate proximal endothelium and increase leukocyte extravasation. It has been proposed that secondary chemoattractants are secreted into sequential waves and LTB4 is the first secondary chemoattractant released at a site of inflammation (Afonso et al. 2012).

LTB4 has been originally discovered as a strong chemotactic factor, which exerts PMN activating abilities and plays a crucial role in neutrophil migration. Several studies have reported elevated levels of LTB4 also in bronchoalveolar lavage (BAL) fluid from human patients with acute respiratory distress syndrome (ARDS). Moreover, the evaluation of LTB4 has been described as useful prognostic indices in human patients with early phase ARDS after admission to the intensive care unit, but there is no data using a horse model of asthma according to the review made by Störmann et al. (2017).

The immunologic background of severe equine asthma remains not fully elucidated despite many studies on the pathogenesis and the therapeutic approach to exacerbation. Therefore, multiple studies have been performed to establish further inflammatory markers for equine respiratory diseases, but there are no data on the role of LTB4 in these diseases yet (Barton & Gehlen 2016).

Thus, this study aimed to evaluate the LTB4 concentration in the bronchoalveolar lavage fluid (BALF) of horses with and without pulmonary inflammatory disease.

MATERIALS AND METHODS

Ethical statement. This study was submitted to the Ethics Committee on Animal Experiments of the "Universidade Federal Rural do Rio de Janeiro" (UFRRJ) and approved under the identification number 2927310316. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Animals. Thirty-five mixed breed horses, males and females, with ages from two to 24 years were studied. The horses were raised, extensively, at grass covered pasture (*Brachiaria* spp. and *Andropogon bicornis*) without shelter and areas of dry dirt, with minimal exposure to dust, with no exercise training, with supplementary feed consisting of two kilogram per animal of hard commercial feed and mineral salt offered in troughs covered once a day and *ad libitum* water. The horses were used for practical classes at UFRRJ and were not used for work purposes. The animals were periodically dewormed and vaccinated against tetanus, influenza, rabies, and encephalomyelitis East and West. The horses were clinically evaluated which included evaluation of vital parameters, lung percussion and thoracic auscultation with a re-breathing bag (Jorge et al. 2014). None of the horses showed dyspnea at rest or nasal secretions at the time of the study. Blood and BALF samples were collected during the month of January of 2016 (summer in

Southern Hemisphere). The animals were grouped by two different criteria for statistical analysis of data:

1) Values for BALF cytology according to Mazan (2010): Healthy Group (HG) - composed by 13 horses which the values for BALF cytology were neutrophils <5% and eosinophils <1%; Sick Group (SG) - composed by 22 horses which the values for BALF cytology were neutrophils ≥5% and/or eosinophils ≥1%.

2) Detection of LTB4 in BALF: Leukotriene Group (LKT) - composed by six horses where it was possible to quantify the concentration of this inflammatory mediator; No Leukotriene Group (NoLKT) - composed by 29 horses where LTB4 concentration was below the detection limit of the ELISA kit used for this analysis.

Endoscopic examination. A flexible fiberscope was introduced in one of the nostrils and advanced until the tracheal bifurcation (carina) for evaluation of tracheal secretions.

BALF collection. Horses were restrained and sedated with detomidine 1% (0.015mg/kg) associated with butorphanol tartaratec (0.03mg/kg) intravenously before the procedure. To anesthetize the airways, including the larynx, tracheal lumen, and the first bronchial segment, 0.5% lidocaine was instilled by introducing a bronchoalveolar lavage catheter into one of the animal's nostrils until catheter passage resistance was achieved, which signaled that the primary bronchus was reached. After this the catheter's cuff was inflated so that it was stuck to the bronchial segment to infuse the sterile saline solution. BALF was obtained through infusion of two aliquots of 250mL of warm physiological isotonic saline solution (37^0C), followed by immediate aspiration of each aliquot. Samples were immediately cooled on ice bath and kept refrigerated until processing within four hours after collection.

Smears for cytology were prepared by cytocentrifugation, followed by fixation with methanol and staining with Giemsa. The slides were examined through an optical microscope with 1000x magnification and the 500-cell count was performed excluding epithelial cells. The cell counts on the slides were made by the same observer.

LTB4 concentration in BALF. The cooled equine BALF samples were centrifuged at 269xg for 10min and the supernatant stored at -80°C until the sample analysis for LTB4 concentrations using the leukotriene B4 ELISA Kit according to manufacturer's instructions (Cayman Chemical©, Michigan, USA) at the Laboratory of Inflammation of the Institute of Biophysics Carlos Chagas of the "Universidade Federal do Rio de Janeiro".

Statistics. Statistical software IBM® SPSS Statistics 25 was used to perform the Mann-Whitney-U test to identify significant differences ($p<0.05$) in mean count of Neutrophils, Eosinophils, Macrophages present in equine BALF, age of animals and LTB4 concentration between HG *versus* SG using and significant differences ($p<0.05$) in mean count of neutrophils, eosinophils, macrophages present in equine BALF and age of animals between LKT *versus* NoLKT groups.

RESULTS AND DISCUSSION

Of the 35 horses, it was possible to quantify LTB4 in six animals (17.14%) and of these animals four were sick and two healthy. To our knowledge, this is the first report of detection of LTB4 in equine BALF. The results of the animals analyzed in this study are detailed in the Table 1.

Unexpectedly, there was no significant differences ($p>0.05$) between HG and SG neither in mean count of Neutrophils, Eosinophils, Macrophages nor LTB4 concentration in BALF. There was also no effect of age on the occurrence of pulmonary inflammation of horses in this study.

Comparing the groups separated by the LTB4 detection criterion in BALF there was also no significant differences ($p>0.05$) between LKT Group and NoLKT Group in mean count of neutrophils, eosinophils and macrophages present in equine BALF, but there was significant difference ($U=42.000$, $p<0.05$) of effect of age on the LTB4 detection in equine BALF. Younger animals (5/6) were those where it was possible to detect more animals with LTB4 values in BALF than older animals (1/6). This could be explained because young animals are able to respond to immunological challenges, since their leukocytes are present in adequate numbers and are able to respond to antigens satisfactorily, producing the necessary inflammatory mediators (Butcher et al. 2000).

It is worth mentioning that the highest concentration of LTB4 detected was in the two-year-old foal. On the other hand, elderly individuals have higher rates of mortality and morbidity due to infectious diseases. Although the elderly patients have normal numbers of neutrophils in their blood, a decline in their function has been demonstrated through the use of various stimuli. Neutrophils are the leukocytes that respond more rapidly to pathogen invasion, and it is known that there is a decline in their age-related function (immunosenescense) and this may be partly responsible for the greater susceptibility of the elderly to infections (Wenisch et al. 2000). Albeit limited, current evidence suggests that horses likely undergo similar age-related changes in immune function as that observed in people. Immunosenescence includes age-associated remodeling of the immune system resulting in poor immunity and an exaggerated inflammatory state. Although aged horses appear to generally maintain an adequate protective immunity against pathogens, inflammatory diseases are extremely common in the geriatric equid (McFarlane 2016).LTB4 is a potent attractant for inflammatory cells (neutrophils and eosinophils) and these cells have the ability to generate further quantities of LTB4 and other lipoxygenase products (O'Driscoll et al. 1984). It is noteworthy that the only elderly animal in which it was possible to quantify LTB4 concentration was the one that presented the highest percentage of neutrophils (26.2%), that is, it presented more than 20% of neutrophils, which characterizes it as a severe case of equine asthma (Couëtil et al. 2016). It is necessary to emphasize that Animal 2 also presented a BALF cytology compatible with EAS (10.4% of neutrophils), however the percentage was less than half of the Animal 4 and both horses had similar concentrations of LTB4 which corroborates with that described by Wenisch et al. 2000 and McFarlane 2016.

In later 2017, Störmann et al. evaluated the concentration of LTB4 in polytraumatic pigs and verified significant levels of LTB4 in BALF up to 72 hours after trauma, demonstrating the flashing action of this inflammatory mediator. Therefore, LTB4 could be used as an early marker of inflammatory processes in the respiratory system. Chronic diseases have lower odds of detecting LTB4 in BALF when compared to acute diseases or cases of inflammatory condition exacerbation. Afonso et al. (2012) described leukotriene B4 (LTB4) as the first secondary chemoattractant released at an inflammation site, confirming its rapid action. In agreement with this finding, Antonelli et al. (1994) suggest that LTB4 levels have been shown to correlate with the number of neutrophils recovered from the BAL fluid of human patients with ARDS. Additionally, Ochkur et al. (2013) report that another leukotriene, other

Table 1. Identification, age, mean percentage of BALF cytology (neutrophils, lymphocytes, macrophages and eosinophils), cytological classification and LTB4 concentration in BALF of horses included in this study

	Animal		Age (Years)	Neutrophils (%)	Linphocytes (%)	Macrophages (%)	Eosinophils (%)	LTB4 (pg/mL)
SG	LKT	1	2	5	54.3	40.5	0.2	8.803.022
		2	3	10.4	50	37.4	2.2	3.520.752
		3	3	9.5	27	62.7	0.8	2.487.726
		4	18	26.2	30	37.4	6.4	3.897.825
	NoLKT	5	3	8	44	47.2	0.8	ND
		6	5	5.4	53	40.2	1.4	ND
		7	2	4	56.6	37.8	1.4	ND
		8	7	14.4	42	43.6	0	ND
		9	9	5	57.6	37.4	0	ND
		10	14	12.3	48	39.7	0	ND
		11	10	14.4	51	38.2	0	ND
		12	9	6.6	52.8	40	0.6	ND
		13	13	5.4	59.2	35.2	0.2	ND
		14	14	7.8	68	23.2	1	ND
		15	16	7.6	51.6	33	7.8	ND
		16	16	11.8	29	59.2	0	ND
		17	16	10	49.6	40.2	0.2	ND
		18	19	12	42	36.8	0.2	ND
		19	19	12.8	37.4	49.6	0.2	ND
		20	3	5.6	55.6	38.8	0	ND
		21	24	14	45.7	40.3	0	ND
		22	13	5.4	56.4	37.8	0.4	ND
HG	LKT	23	3	3	54.4	42.4	0	0.8591229
		24	5	3.2	58	38.8	0	1.136.526
	NoLKT	25	4	4.8	50.6	44.6	0	ND
		26	6	2.8	67.6	29.4	0.2	ND
		27	8	3.6	60.4	35.8	0.2	ND
		28	8	3.6	52.2	44.2	0	ND
		29	12	1.4	43.2	49	0.4	ND
		30	9	3	48.2	48	0.4	ND
		31	9	3.6	36.6	59	0.8	ND
		32	17	4.8	41.3	53.9	0	ND
		33	17	3.2	63.2	33.2	0.4	ND
		34	3	2.6	36.4	61	0	ND
		35	3	4.4	48.4	46.8	0	ND

SG = sick group, HG = healthy group, LKT = possible to quantify the concentration of LTB4, NoLKT = not possible to quantify the concentration of LTB4.

than LTB4, plays an important role in pulmonary remodeling in chronic respiratory processes and its study in mice reveals that the progressive pulmonary fibrosis appears to occur as a consequence of leukotriene-mediated activities. In particular, this chronic inflammatory metric was shown to be a function of cys-leukotrienes, and not LTB4. It could explain why our experiment were not able to detect LTB4 in all horses with BALF cytology compatible with lower respiratory tract inflammation, because it is believed that animals with inflammatory BALF cytology compatible with pulmonary inflammatory disease are in a chronic or remission condition situations where LTB4 would no longer be the chemotactic protagonist.

Another factor contributing to the non-detection of LTB4 is that although the ELISA has the economic advantage, it also has its limitations. The use of the ELISA has bottlenecks: ELISA technique certainly has its specificity limitations due to used detection antibodies, corroborating with what has already been described by Störmann et al. (2017) when they recommend, for future studies, that samples should be analyzed by liquid chromatography-ultraviolet-tandem mass spectrometry technique, since is more reliable and sensitive method to quantity LTB4.

CONCLUSION

In conclusion, it was possible to detect the concentration of LTB4 in the equine LBA. It was also concluded that there was an effect of age on the detection of LTB4 in equine BAL, where LTB4 levels were more easily detected in younger animals than older animals. The results of this study raise the possibility of considering future studies with the objective of establishing the real role and the best moment to detect LTB4 in BALF of the equine asthma syndrome.

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Detection of efflux pump CmeABC in enrofloxacin resistant *Campylobacter* spp. strains isolated from broiler chickens (*Gallus gallus domesticus*) in the state of Rio de Janeiro, Brazil[1]

Regina J. Nascimento[2]*, Beatriz S. Frasão[3], Thomas S. Dias[2], Elmiro R. Nascimento[2], Louise S.B. Tavares[2], Virginia L. Almeida[2] and Maria Helena C. Aquino[2]

ABSTRACT.- Nascimento R.J., Frasão B.S., Dias T.S., Nascimento E.R., Tavares L.S.B., Almeida V.L. & Aquino M.H.C. 2019. **Detection of efflux pump CmeABC in enrofloxacin resistant *Campylobacter* spp. strains isolated from broiler chickens (*Gallus gallus domesticus*) in the state of Rio de Janeiro, Brazil.** *Pesquisa Veterinária Brasileira 39(9):728-733*. Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: reginajulia@gmail.com

Fowls are the main reservoirs of the highly important food-originating pathogen called *Campylobacter* spp. and broilers' meat and byproducts are the main vehicles of this microorganism. Increasing of *Campylobacter* spp. resistant strains to fluorquinolones, an antimicrobial class often employed in poultry farming and in human medicine has become a great concern to poultry breeders. In fact, several studies evaluated increasing bacterial resistance against these antimicrobial agents. The role of CmeABC efflux system has been underscored among the resistance mechanisms in *Campylobacter* spp. to fluorquinolones. This study investigated the occurrence of CmeABC efflux pump in 81 and 78 enrofloxacin resistant strains of *Campylobacter jejuni* and *C. coli* respectively, isolated from broilers collected from six abattoirs situated at São José do Vale do Rio Preto/RJ poultry center and from two commercial abattoirs situated at Metropolitan Region of Rio de Janeiro, from 2013 to 2016. The resistance to enrofloxacin was assessed by agar dilution to determine minimum inhibitory concentration (MIC). The CmeABC efflux system was investigated through the detection of genes genes *cmeA*, *cmeB* and *cmeC* by PCR. The activity of CmeABC efflux pump was investigated in 20 strains by using the efflux pump inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN). The three genes *cmeA*, *cmeB* and *cmeC* were detected in 94.3% of the strains (*C. jejuni* = 80 and *C. coli* = 70), whereas the system was absent or incomplete in 5.7% of strains (*C. jejuni* = 1 and *C. coli* = 8). MIC varied between 0.5µg/ml and 64µg/ml, and 88.7% of strains were enrofloxacin resistant and 11.3% featuring intermediate resistance. The inhibition of the efflux pump by PAβN reduced the MIC to enrofloxacin up to eight times in fifteen strains (75%). These results indicate that this system is frequent and active in *Campylobacter* spp. Resistant strains in the presence of enrofloxacin.

INDEX TERMS: Efflux pump, CmeABC, enrofloxacin, *Campylobacter* spp., broiler chickens, *Gallus gallus domesticus*, Rio de Janeiro, Brazil, resistance, chickens.

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[2] Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. *Corresponding author: reginajulia@gmail.com

[3] Universidade Federal do Oeste da Bahia (UFOB), Colegiado de Medicina Veterinária, Av. 23 de Agosto 872, Barra, BA 7100-000, Brazil.

RESUMO.- [Detecção da bomba de efluxo CmeABC em cepas de *Campylobacter* spp. resistentes à enrofloxacina isoladas de frangos de corte (*Gallus gallus domesticus*) no Estado do Rio de Janeiro, Brasil.] As aves são os principais reservatórios *de Campylobacter* spp., importante patógeno de origem alimentar e a carne de frango e produtos derivados

são os principais veículos desse microrganismo. O aumento de cepas de *Campylobacter* spp. resistentes às fluorquinolonas, uma classe antimicrobiana frequentemente empregada na avicultura e na medicina humana, tornou-se uma grande preocupação para os produtores de aves e vários estudos avaliaram o aumento da resistência bacteriana a esses antimicrobianos. O papel do sistema de efluxo CmeABC tem sido enfatizado entre os mecanismos de resistência em *Campylobacter* spp. à fluorquinolonas. O presente estudo investigou a ocorrência da bomba de efluxo CmeABC em 81 cepas de *Campylobacter jejuni* e 78 cepas de *Campylobacter coli* resistentes à enrofloxacina, isoladas de frangos de corte coletados em seis abatedouros situados no polo avícola de São José do Rio Preto/RJ e de dois abatedouros comerciais situados na Região Metropolitana do Rio de Janeiro, de 2013 a 2016. A resistência à enrofloxacina foi avaliada pelo método de diluição em ágar para determinar a concentração inibitória mínima (CIM). O sistema de efluxo CmeABC foi investigado através da detecção dos genes *cmeA, cmeB e cmeC* por PCR. A atividade da bomba de efluxo CmeABC foi investigada em 20 cepas utilizando o inibidor da bomba de efluxo Phenylalanine-Arginine β-Naftilamida (PAβN). Os três genes *cmeA, cmeB e cmeC* foram detectados em 94,3% das cepas (C. jejuni = 80 e C. coli = 70), enquanto o sistema estava ausente ou incompleto em 5,7% das cepas (*C. jejuni* = 1 e *C coli* = 8). A CIM variou entre 0,5µg/ml e 64µg/ml e 88,7% das cepas foram resistentes à enrofloxacina, enquanto 11,3% apresentaram resistência intermediária. A inibição da bomba de efluxo pelo PAβN reduziu a CIM da enrofloxacina até oito vezes em quinze cepas (75%). Estes resultados indicam que este sistema é frequente e ativo em cepas resistentes de *Campylobacter* spp. na presença de enrofloxacina.

TERMOS DE INDEXAÇÃO: Bomba de efluxo, CmeABC, cepas, *Campylobacter* spp., enrofloxacina, frangos de corte, *Gallus gallus domesticus*, Rio de Janeiro, Brasil, resistência.

INTRODUCTION

The food-borne zoonotic pathogen *Campylobacter* spp. is one of the main gastroenteritis agents worldwide, especially in developing countries (Zhou et al. 2016). In the EU, 246.307 cases of campylobacteriosis were reported in 2016, featuring the most relevant cause of gastroenteritis since 2005 (EFSA & ECDC 2017). In the USA, *Campylobacter* was the most registered cause of bacterial gastroenteritis, with an estimate of 1.3 million cases a year (CDC 2017). Most campylobacteriosis cases are associated to the ingestion of raw and undercooked chicken meat or to cross-contaminated meat. Its prevalence in broilers' carcasses ranges from 0.29% to 96.7% (Aquino et al. 2002, Garin et al. 2012, Wang et al. 2013).

In developing countries, such as Brazil, information on outbreaks is often incomplete and diarrheal diseases are endemic. Although Brazil is the largest exporter of chicken meat in the world, the presence of *Campylobacter* spp. is not investigated in most cases of human bacterial gastroenteritis. This is probably due to the particularities of its isolation and characterization methodology, which is different from that applied to detect enteropathogenic bacteria, such as *Escherichia coli*, *Salmonella* and *Shigella* (Panzenhagen et al. 2016a).

Resistance to fluoroquinolones in *Campylobacter* spp. strains has been reported in several countries, with different results in the treatment of infections (Helms et al. 2005). The first reported resistance case in human treatment for *C. jejuni* occurred when enrofloxacin was used in the treatment of broilers in Holland in the winter of 1987 (Endtz et al. 1991).

Mutation in the Quinolones Resistance-Determining Region (QRDR) of gene *gyrA*, which codifies for subunit A of the DNA *gyrase* enzyme, substitutes Tre-86-Ile and is the main resistance mechanism to fluoroquinolones (Wieczorek & Osek 2013). However, several studies show that mutation in QRDR may not be the sole mechanism in the resistance to these drugs and reveal that efflux pump codified by *cmeA*, *cmeB* and *cmeC* genes is an important factor in *Campylobacter* spp. resistance to several antimicrobial agents (Poole 2005, Iovine 2013, Wieczorek & Osek 2013), alone or coupled to other mechanisms.

The inhibitor of efflux pump Phenylalanine-Arginine β-Naphthylamide (PAβN) is efficient against several Gram-negative bacteria and compromises nutrient uptake and excretion of toxic compounds through its inhibition. Several authors also underscore that the inhibition of the CmeABC efflux system by PAβN increases the susceptibility of *C. jejuni* to different antibiotics, including macrolides and fluoroquinolones, which are important drugs for the treatment of human campylobacteriosis (Mamelli et al. 2003, Chollet et al. 2004, Hasdemir et al. 2004, Saenz et al. 2004).

This study investigated the presence of the CmeABC efflux system and its activity in enrofloxacin resistant *C. jejuni* and *C. coli* strains isolated from broilers in the state of Rio de Janeiro, Brazil.

MATERIALS E METHODS

Sample collection and species identification. Strains were obtained from samples of broilers' intestines, retrieved immediately after evisceration collected from six abattoirs situated at São José do Vale do Rio Preto poultry center with state inspection, and from two commercial abattoirs of State of Rio de Janeiro, from 2013 to 2016. In each collect, 10 broilers' intestines were collected and taken to the laboratory, for processing at the same day. Swabs with caecum material were diluted in 2mL of sterilized distilled water, 0.30ml was filtered by cellulose acetate membrane (Sartorius) (0.65µm), and spreaded on Columbia agar plates, supplemented with activated coal (0.4%) and CAMPYLOFAR® (CEFAR) (Aquino et al. 2002). Plates were incubated at 37°C for 48 hours in microaerophilic conditions. Suspect colonies were confirmed by PCR technique (Harmon et al. 1997) and *Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559 strains were employed as positive control.

Detection of antibiotic resistance genes. Eighty-one *Campylobacter jejuni* strains and 78 *C. coli* strains, classified as resistant to enrofloxacina by the agar dilution assay (CLSI 2013),were investigated to detect the CmeABC efflux system. Genes *cmeA*, *cmeB* and *cmeC* were detected by PCR, following Lin et al. (2002) and Obeng et al. (2012) as described: 25uL with 2µl of sample DNA; 2.5µl of 10X PCR Buffer; 0.2mmol l^{-1} of deoxyribonucleotide phosphates (dATP, dCTP, dGTP and dTTP); 0.4µmol l^{-1} of each starter (Table 1); 2.5U of Taq polymerase (Ludwig, Alvorada, Brasil) were employed and Thermal Cycler (ThermoElectron Corporation - Px2 ThermalCycler) was used to run the PCR. Initial denaturalization was undertaken at 96°C for 1 min, followed by 30 cycles with denaturalization for 30 seconds at 94°C; annealing temperature at 54°C for 45 seconds; at 72°C for 1 minute; final extension at 72°C for 5 min. Table 1 provides sequences of primers. For the visualization

Table 1. Primers used in the PCR to detect the CmeABC efflux system in *Campylobacter* strains

Target gene	Primer sequence (5′-3′)		Amplicon size (bp)	Reference
	Forward	Reverse		
cmeA	TTTGGATCCTTGATGGCTAAGGCAACTTTC	CTCCAATTTCTTAAGCTTCGCTACCAA	771	(Lin et al. 2002)
cmeB C. jejuni	GGTACAGATCCTGATCAAGCC	AGGAATAAGTGTTGCACGGAAATT	820	(Lin et al. 2002)
cmeB C. coli	TCCTAGCAGCACAATATG	AGCTTCGATAGCTGCATC	241	(Obeng et al. 2012)
cmeC	GCTTGGATCCTTATCTTGGGAAAAA	TTTTTAAAGCTTTAAGGTAATTTTCTT	624	(Lin et al. 2002)

of the PCR product, 5µL of amplicon was performed in a horizontal electrophoresis tank 'Electrophoresis Cell (BioAmérica) at Power Pac 300 (Bio-Rad) in agar gel 1.5%, buffer TBE (1.0M Tris, 0.01M boric acid, 0.01M EDTA, pH 8.0) (Ludwig, Alvorada, Brasil), and 1µlGelRed with 1µl loading buffer. Image was visualized and photographed in ultraviolet transilluminator (Nova Instruments).

Antibiotic susceptibility test. Resistance to enrofloxacin was investigated by agar dilution method to determine Minimum Inhibitory Concentration (MIC) (CLSI 2013). Suspension of inoculated *Campylobacter* was adjusted to turbidity, equivalent at McFarland 0.5 standard. Mueller Hinton agar plates were supplemented with sheep blood (7%) and enrofloxacin (64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.25µg/ml, and 0.125µg/ml) to determine MIC. The plates were incubated at 37°C for 48 hours under microaerophilic conditions. The strains were classified according to the MIC value (µg/ml) as resistant (≥2µg/ml), intermediate (1µg/ml-0.5µg/ml) or susceptible (≤0.25µg/ml).

Effect of efflux pump inhibitor on antimicrobial resistance. The twenty most recently isolated strains harboring the three genes (by *cmeA*, *cmeB* and *cmeC*) had the activity of CmeABC efflux investigated, by efflux pump inhibitor Phenylalanine-Arginine β-Naphthylamide (PAβN) (MP Biomedicals, Santa Ana, California, USA). To determine the efflux pump activity, MIC was determined by agar dilution method with and without the inhibitor PaβN according to Hungaro et al. (2015). Mueller Hinton agar plates were supplemented with sheep blood (7%), inhibitor PAβN (5µg ml^{-1}) and enrofloxacin (64µg/ml, 32µg/ml, 16µg/ml, 8µg/ml, 4µg/ml, 2µg/ml, 1µg/ml, 0.5µg/ml, 0.25µg/ml, 0.125µg/ml). The plates were incubated at 37°C for 48 hours under microaerophilic conditions and changes in the MIC value were investigated.

RESULTS AND DISCUSSION

The three genes, *cmeA*, *cmeB* and *cmeC,* were detected in 94.3% (150) of 159 strains. Nine strains (5.7%) failed to have the full efflux system as described below: in one *Campylobacter coli* strain, the three genes were absent and in five *C. coli* strains the *cmeB* was not detected; the genes *cmeB* and *cmeC* were not detected in one *C. coli* strain and *cmeA* was not detected in one *Campylobacter jejuni* and in one *C. coli* strain.

In this study, the efflux pump system was observed in most *C. jejuni* e *C. coli* strains isolated from broilers. These results are in agreement with those of others authors from Brazil and other countries (Hungaro et al. 2015, Lin et al. 2002, Van Deun et al. 2007, Cantero et al. 2018) which frequently observed the presence of this efflux system in *Campylobacter* strains. Of the nine strains in which the full efflux system was not detected, one was isolated from a commercial abattoir and eight were from the poultry center located São José do Vale do Rio Preto.

Two efflux systems involved in the resistance mechanism of *Campylobacter* have been well characterized, namely, *Campylobacter* multidrug efflux CmeABC and CmeDEF, belonging to the proton motive force-dependent group, and Resistance-Nodulation-Division family (RND) (Jeon et al. 2011). The family RND is usually registered in Gram-negative bacteria and its efflux system works by a tripartite system that includes a periplasmatic membrane fusion protein, an inner membrane drug transporter and an outer membrane protein, which are codified, respectively, by genes *cmeA, cmeB* and *cmeC* in *Campylobacter* (Lin et al. 2005, Poole 2005). The three proteins are codified by a three-gene operon, (CmeABC), working together to expel toxic substrates from the interior of the bacterial cell (Lin et al. 2002).

Efflux systems in *Campylobacter* participate in the uptake of essential nutrients and ions, in the excretion of bacterial metabolism products and toxic substances. They also participate in the communication processes between cells and the environment. They have also been studied as a potential target to reduce resistance to antibiotics in *Campylobacter* spp. and as an alternative strategy to limit contaminations and prevent infections (Možina et al. 2011, Nikaido & Jean-Marie 2012).

Resistance-nodulation-division (RND) efflux systems contribute towards *Campylobacter*'s intrinsic resistance for a wide range of structurally non-related antimicrobial agents. They may acquire resistance to fluoroquinolones by super-expression of efflux proteins or by synergic interaction with resistance mechanisms to non-efflux fluoroquinolones, such as mutations in gene *gyr*A (Lin et al. 2002, Luo et al. 2003). Luangtongkum et al. (2009) showed that CmeABC system works synergically with *gyr*A in the mediation of resistance to fluoroquinolones, whereas strains resistant to fluoroquinolones without Tre-86-Ile mutation have already been reported (Hungaro et al. 2015).

Minimum Inhibitory Concentration (MIC) in this study ranged from 0.5 to 64µg/mL, and 88.7% of the strains were resistant, whereas 11.3% showed intermediate resistance. The highest levels of resistance were observed in abattoir 3 (Table 2), a commercial abattoir, located at metropolitan region of Rio de Janeiro In Brazil, enrofloxacin is frequently employed in poultry breeding, and there is no effective control by regulatory agencies of its use in small producers, as may be verified in the report of the State Program for the Control of Veterinary Medicine Wastes in Animal-derived Food (PAMvet) in the state of Paraná, Brazil (Machinski Junior et al. 2005). In fact, Panzenhagen et al. (2016b) registered enrofloxacin wastes in 72.2% of broilers' samples collected in the state of Rio de Janeiro, analyzed by Enzyme-Linked Immunosorbent Assay (ELISA), albeit at rates lower than the Maximum Waste Limits. In Spain, Cantero et al. (2018) reported that

Table 2. Strains, origin and determination of MIC with and without PAβN inhibitor in 20 *Campylobacter* spp. strains

Campylobacter strains	Source	*CmeA*	*CmeB*	*CmeC*	MIC (μg/mL)	MIC with PAβN (μg/mL)
C. coli	Abattoir 1	+	+	+	4(R)	1(I)
C. coli	Abattoir 1	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	1(I)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 2	+	+	+	1(I)	1(I)
C. coli	Abattoir 2	+	+	+	2(R)	1(I)
C. coli	Abattoir 3	+	+	+	64(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	8(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	1(I)
C. coli	Abattoir 3	+	+	+	8(R)	8(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	8(R)	4(R)
C. coli	Abattoir 3	+	+	+	16(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	2(R)
C. jejuni	Abattoir 3	+	+	+	4(R)	4(R)
C. jejuni	Abattoir 3	+	+	+	16(R)	8(R)

Abattoir 1 = located in São José do Vale do Rio Preto, Abattoir 2 and 3 = located in Metropolitan Region of Rio de Janeiro, R = resistant strain, I = intermediate resistance.

isolated strains of broilers were predominantly resistant to quinolones, perhaps due to the frequent use of enrofloxacin in poultry breeding. The 2016 EU´s report similarly described resistance to antimicrobial agents in zoonotic bacteria and in indicating bacteria from humans, animals and food. In fact, *Campylobacter* strains isolated from humans and broilers showed high to extremely high resistance to ciprofloxacin (EFSA & ECDC 2018).High minimum inhibitory concentration for enrofloxacin in broiler-derived strains in this study may compromise the efficaciousness of fluoroquinolones in the treatment of human infections caused by poultry-derived strains.

MIC varied from 8 to 32μg/mL in strains without *cmeA* or *cmeC,* whereas resistance ranged from 0.5 to 32μg/mL in 7 strains in which the gene *cmeB* was not detected. These findings suggest the participation of another resistance mechanism besides the efflux pump. The lack of genes *cmeA* and *cmeC* in a study by Hungaro et al. (2015) failed to affect MIC, whereas Pumbwe & Piddock (2002) reported that the absence of *cmeB* made possible the recovery of levels of sensitiveness. The gene *cmeB* codifies the internal membrane which transports drugs and it is fundamental for the functioning of the CmeABC efflux system. Our study investigated the activity of efflux pump with inhibitor PAβN in *Campylobacter* spp. strains with complete efflux system and revealed a decrease in MIC of enrofloxacin from two to eight times in 15 (75%) strains. Despite the MIC decreasing, eight strains remained resistant and seven strains showed intermediate resistance (Table 2). No change in the MIC of 5 (25%) strains with the complete efflux system was observed, suggesting that the efflux system was inactive.

Effective participation of the CmeABC efflux system in the *Campylobacter* fluoroquinolone resistance has not always been reported. Mavri & Možina (2012) reported MIC reductions for ciprofloxacin with PAβN inhibitor in only 50% of strains derived from broilers, pigs, humans and surface water. Corcoran et al. (2005) demonstrated that PAβN inhibitor did not cause significant decrease in resistance to fluoroquinolones in *Campylobacter* spp. isolates from broilers and humans. On the other hand, Kurinčič et al. (2012) reported MIC decrease in fluoroquinolones and the reestablishment of susceptibility with PAβN inhibitor in *Campylobacter* isolates from food, animals, water and humans. These results indicate that the efflux mechanism is involved in the resistance to enrofloxacin in *Campylobacter*, but not as a single mechanism. In fact, in our study, several resistant strains did not have the full CmeABC efflux system or the activity of the efflux pump was not reduced by the use of PAβN inhibitor.

Several studies have been undertaken recently on natural compounds which may inhibit the efflux system of bacteria (Chérigo et al. 2009, Fadli et al.2011, Ramalhete et al. 2011, Roy et al. 2012, Možina et al.2018). Due to the crucial role of the CmeABC system in the adaptation of *Campylobacter* spp. in the intestine tract, it has been suggested that this would be a proper target for the control of infections by *Campylobacter* spp. The inhibition of efflux systems in *C. jejuni* may be a new approach to decrease resistance to antibiotics and prevent infection by *Campylobacter* spp. in human beings and animal reservoirs. The employment of *CmeABC* inhibitors as food additives in poultry breeding systems to prevent or decrease intestine colonization and reduce the dissemination of *Campylobacter* resistant strains may be considered an advance in the use of inhibitors. In fact, many cases of human campylobacteriosis are attributed to the intake of raw or undercooked chicken meat (Hungaro et al. 2015, Možina et al. 2018). Further, several regulating mechanisms that change CmeABC expression have been described (Lin et al. 2005) and information on them and knowledge on the structure, functions and regulation of other efflux pumps in *Campylobacter* may identify new targets for the therapeutic intervention of *Campylobacter* (Možina et al. 2018).

CONCLUSIONS

Our findings suggest that CmeABC efflux system is an important mechanism in the *Campylobacter* resistance to enrofloxacin .However, the lack of a full efflux system in resistant strains and the MIC maintenance in the presence of the inhibitor PAβN indicates the participation of other mechanisms or a synergic activity in the resistance of enrofloxacin.

The use of efflux pump inhibitors may offer perspectives to reduce antimicrobial resistance and the colonization of *Campylobacter* spp. in broilers.

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Chemical composition and antimicrobial activity of two extract of propolis against isolates of *Staphylococcus* spp. and multiresistant bacterials[1]

Jarbas F. Amarante[2], Márcia F. Ribeiro[3], Mateus M. Costa[2], Fredson G. Menezes[2], Tania M.S. Silva[4], Talita A.B. Amarante[5], Adriana Gradela[2]* and Liliane M.D. Moura[2]

ABSTRACT.- Amarante J.F., Ribeiro M.F., Costa M.M., Menezes F.G., Silva T.M.S., Amarante T.A.B., Gradela A. & Moura L.M.D. 2019. **Chemical composition and antimicrobial activity of two extract of propolis against isolates of *Staphylococcus* spp. and multiresistant bacterials.** *Pesquisa Veterinária Brasileira 39(9):734-743.* 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

There is a growing need to discover and develop alternative therapies for the treatment of mastitis caused by *Staphylococcus* spp. and multidrug-resistant bacterial infections. This study examined the chemical composition and antimicrobial potential of two propolis extracts (EPA and EPB) against seventy-seven isolates of *Staphylococcus* spp. obtained from subclinical bovine mastitis; three clinical strains of MRSA and two from clinical strains of *S. aureus* ATCC, identified as *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923. The total phenolic content was determined by the Folin-Ciocalteau method, the total flavonoid content by the Dowd method and the phenolic profile was quantified by HPLC-DAD. The MBC values of the extracts were evaluated by broth microdilution method. The amount of total phenolic and flavonoid compounds was higher in EPA than EPB. Both extracts revealed the presence of caffeic, coumaric, cinnamic, ferulic and 3,4-dihydroxybenzoic acids, with higher concentrations of coumaric and cinnamic acids. *Staphylococcus* spp. isolates were susceptible to EPA (90.9%), EPB (83.1%) and oxacillin (80.5%). The oxacillin susceptible isolates were also susceptible to EPA (70.1%) and EPB (80.6%), whereas those oxacillin-resistant strains were also susceptible to EPA (40.0%) and to EPB (26.7%). MBC ranged from 34.3 to 68.7µm/mL for EPA and from 68.7 to 137.5µg/mL for EPB. Both extracts inhibited significantly (100%) the clinical strains of MRSA, *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 at the concentration of 68.7µg/mL. It is concluded that both extracts of propolis, whose main constituents are coumaric and cinnamic acids, have high antimicrobial activity against the microorganisms studied, and EPA also against oxacillin-resistant strains. These findings reinforce its potential use for the treatment of bovine mastitis.

INDEX TERMS: Chemical composition, antimicrobial activity, extract of propolis, *Staphylococcus* spp., multiresistant bacterials, phenolic compounds, microdilution, oxacillin-resistant *Staphylococcus aureus*, propolis, bacterioses.

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[2] Universidade Federal do Vale do São Francisco (Univasf), Rodovia 407 Km 12, Lote 543, Projeto Nilo Coelho C1, Petrolina, PE 56300-000, Brazil. *Corresponding author: agradela@hotmail.com

[3] Centro de Pesquisa Agropecuária do Trópico Semi-Árido (CPATSA), Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Rodovia BR-428, Km 152, Zona Rural, Petrolina, PE 56302-970.

[4] Departamento de Ciências Moleculares, Universidade Federal Rural de Pernambuco (UFRPE), Rua Manuel de Medeiros s/n, Dois Irmãos, Recife, PE 52171-900, Brazil.

[5] Departamento de Ciências Humanas (DCH), Universidade do Estado da Bahia (UNEB), R. Silveira Martins 2555, Cabula, Salvador, BA 41150-000, Brazil.

RESUMO.- [**Composição química e atividade antimicrobiana de dois extratos de própolis contra isolados de *Staphylococcus* spp. e bactérias multirresistentes.**] É cada vez mais oportuna a necessidade de descobrir e desenvolver terapias alternativas para tratamento da mastite causada por *Staphylococcus* spp. e de infecções bacterianas multirresistentes. Este estudo examinou a composição química e o potencial antimicrobiano de dois extratos etanólicos de própolis (EPA e EPB) contra setenta e sete isolados de *Staphylococcus* spp. obtidos a partir de mastite bovina subclínica; três estirpes clínicas de MRSA e duas de linhagens clínicas de *S. aureus* ATCC, identificadas como, *S. aureus* ATCC 6538 e *S. aureus* ATCC 25923, ambas metacilina resistentes. O teor total de fenólicos foi determinado pelo método de Folin-Ciocalteau, o teor de flavonoides totais pelo método Dowd e o perfil fenólico foi quantificado por HPLC-DAD. CBM dos extratos foi avaliada pelo método de microdiluição em caldo. A quantidade total de compostos fenólicos e flavonoides foi maior no EPA do que no EPB. Ambos os extratos revelaram a presença dos ácidos cafeico, cumárico, cinâmico, ferúlico e 3,4-di-hidroxibenzóico, com maiores concentrações de ácidos cumárico e cinâmico. Os isolados de *Staphylococcus* spp. foram sensíveis a EPA (90,9%), EPB (83,1%) e oxacilina (80,5%). Os isolados suscetíveis à oxacilina também foram suscetíveis ao EPA (70,1%) e ao EPB (80,6%), enquanto os do resistente à oxacilina foram suscetíveis ao EPA (40,0%) e ao EPB (26,7%). MBC variou de 34,3 a 68,7µm/mL para EPA e de 68,7 a 137,5µg/mL para EPB. Ambos os extratos inibiram significativamente (100%) as linhagens clínicas de MRSA, *S. aureus* ATCC 6538 e *S. aureus* ATCC 25923 na concentração de 68,7µg/mL. Conclui-se que os extratos etanólicos da própolis, cujos principais constituintes são os ácidos cumário e cinâmico, possuem atividade antimicrobiana contra os micro-organismos estudados, e o EPA também contra as cepas resistentes à oxacilina. Estes achados reforçam seu potencial uso para o tratamento da mastite bovina.

TERMOS DE INDEXAÇÃO: Composição química, atividade antimicrobiana, extrato de própolis, *Staphylococcus* spp., bactérias multirresistentes, compostos fenólicos, microdiluição, *Staphylococcus aureus* oxacilina-resistente, própolis, bacterioses.

INTRODUCTION

In Brazil, milk production is a very important segment, totaling 25.4 billion liters annually. Mastitis is the main disease that affects dairy production, with a prevalence of 48.64% in the subclinical form (Acosta et al. 2016), causing changes in the physical-chemical composition and cellularity of the milk, resulting in high economic damages with the reduction in milk production and significant effects on public health (Langoni 2000, Ribeiro 2008).

Approximately 140 different etiological agents may cause bovine mastitis, mainly in the subclinical form, of which the most prevalent contagious microorganisms have been *Staphylococcus aureus*, *Streptococcus* spp., *Streptococcus agalactiae*, *Staphylococcus* spp. and *Corynebacterium bovis*, in pure culture or in association (Costa 2001, Santos & Fonseca 2007, Ribeiro et al. 2009, Martins et al. 2010, Peixoto et al. 2012, Saeki et al. 2012). *S. aureus* is recognized worldwide as a cause of several purulent diseases in humans and animals (Bean & Griffin 1990) and also an important cause of food poisoning in humans (Omoe et al. 2002). Additionally this, it shows persistence in the mammary tissue, due to the characteristics of its virulence (Dos Santos et al. 2003) and the appearance of resistant strains by the inadequate use of antibiotics in the treatment of diseases (Barberio et al. 2002, Hogeveen et al. 2011). Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are one of the most prevalent in cases of mastitis and have as main characteristic to be multiresistant to the antimicrobials of the beta-lactam group (Freitas et al. 2005).

One of the main tools for the control and treatment of mastitis is antimicrobial therapy (Erskine et al. 2003), but its efficiency is compromised in cases of antimicrobial resistance (Russi et al. 2008, Shi et al. 2010, Spohr et al. 2011). In addition, the use of antimicrobials causes severe economic losses due to the discarding of animals (Silva et al. 2004) and milk (Nero et al. 2007), reduction of milk production and drug costs (Vianni & Lázaro 2003).

The use of phytotherapics has been shown to be a very useful tool for the prevention and treatment of mastitis, as well as avoiding the elimination of residues in milk (Schiavon et al. 2011). In this sense, propolis, a resinous product produced by honey bees, is being used in the treatment of human and animal diseases (Coelho et al. 2010) and has been shown to be a viable and quite promising alternative in the treatment of infections caused by *S. aureus* (Pinto et al. 2001, Endler et al. 2003, Auricchio et al. 2006, Zeighampour et al. 2014, Shahbaz et al. 2015, Chen et al. 2018) due to its proven antibacterial activity (Barbosa et al. 2009, Araujo & Marcucci 2011). However the results using propolis are still conflicting (Pinto et al. 2001, Loguercio et al. 2006, Peixoto et al. 2012), due to its distinct and complex chemical constitution, necessitating the development of further research (Lustosa et al. 2008, Rufatto et al. 2017). In addition, there are few conclusive results regarding their action against MRSA, that play an important role in nosocomial infections (Taubes 2008) and a serious problem on a global scale (Astani et al. 2013). Brazil is the third largest producer of propolis, contributing 10-15% of the worldwide production (Pereira et al. 2002), for this reason studies about the antibacterial activity of Brazilian propolis would contribute to further increase this participation.

The present study investigated the chemical composition and the antimicrobial activity of two extracts of propolis against isolates of *Staphylococcus* spp. obtained from subclinical bovine mastitis and also clinical strains of MRSA and *Sthaplococcus aureus* ATCC 6538 and ATCC 25923.

MATERIALS AND METHODS

Propolis extracts. The bacterial isolates were tested against two commercial extracts of propolis identified as extract of propolis A (EPA) and extract of propolis B (EPB). The EPA was an 11% green propolis extract (Apis Flora®) originating from the Vassourinha do campo (*Baccharis dracunculifolia*) and obtained from hives of Jundiai, state of São Paulo. It consists of the following ingredients: green propolis, neutral alcohol (ethanol) 95.1% food grade and purified water. The EPB was also in 11% neutral propolis and alcohol solution (Santa Bárbara®). It was obtained from hives in the State of Bahia, containing in its composition 55% plant resins; 30% beeswax; 8 to 10% of essential oils; and 5% pollen. Both extracts had its chemical composition analysed at the Laboratory of Natural Products of the "Universidade Federal Rural de Pernambuco" (UFRPE), in Recife, Pernambuco.

Determination of total phenolic content. Quantification of phenolic compounds in the EPA and EPB was performed by the Folin-Ciocalteau method using gallic acid as the standard (Singleton & Rossi Junior 1965). The standard curve of gallic acid had five points of concentration (4, 8, 16, 24, 36µg/mL), with a wavelength of 760nm, with Y=0.0064x+0.4174, where y is the absorbance and x is the concentration; R^2=0.9577. Quantification of phenolic compounds in extracts of propolis was performed in triplicate, with the quantity of phenols expressed in mg of gallic acid equivalent per gram of propolis extract, given the dry extract content (Roesler et al. 2007).

Determination of total flavonoids. The total flavonoid content in the EPA and EPB was determined by the adapted Dowd method (Meda et al. 2005), with absorbance readings at 300nm, constructing a standard curve of quercetin in five concentrations (1, 5, 10, 20, 40µg/ml), with Y=0.0198x+0.3552, where "y" is the absorbance and "x" is the concentration; R^2=0.9807. The total flavonoid content was expressed as mg of quercetin equivalent per gram of propolis extract, given their dry extract content, as described by Lee et al. (2003).

Determination of dry residue content. An aliquot of 5 mL of EPA and EPB, free of wax, was transferred to a porcelain capsule in dry form (heated in a laboratory oven at 105°C, for 2h, cooled in a desiccator and weighed), and the whole was taken to the oven preheated to 105°C, where it remained for 2h. After cooling in desiccator, the set was weighed. The process of heating, cooling and weighing the assembly was repeated at intervals of 1h until a constant mass was reached (when the difference between two consecutive weighing did not exceed 5mg). This analysis was realized in triplicate and the dry residue content (soluble solids in methanol) calculated by the ratio of the mass of the residue deposited in the crucible to the initial mass of the extracts of propolis crude corresponding to the aliquot of 5mL in percent (Instituto Adolfo Lutz 1976, Brasil 2001, European Pharmacopoeia 2002).

HPLC-DAD analysis. The High Performance Liquid Chromatography (HPLC) system consisted of two SCL-10Avp solvent pumps, equipped with a SPDM2O diode array detector (HPLC-DAD; Shimadzu, Corp., Kyoto, Japan). Samples were injected into a Rheodyne 7125i type injector with a 20mL capacity loop. Chromatographic separation was done with a C-18 column (25cm x 4.6mm x 5mm, Shimpack CLC-ODS), pre-column C-18 SULPELCO 4.0mm. Water: formic acid (99: 1, solvent A) and methanol (solvent B) were used as the mobile phase and water was used for the acid derivatives: formic acid (95:5, solvent A) and methanol (solvent B). The chromatographic condition was: 0-15min 20% B, 15-20min 30% B, 20-30min 40% B, 30-40min 40% B, with flow rate of 1.0mL/min. For monitoring, the wavelength of 290nm and temperature of 40°C (Dalmora et al. 1997) were used. The identification of phenolics was based on retention times, UV-spectra and chromatographic comparison (co-injection) with authentic markers.

The caffeic, p-coumaric, ferulic, cinnamic, and 3,4-dihydroxybenzoic acids identified in propolis were quantified using the external standard method based on EPAk area. Analyses were made by drawing a calibration curve. To make the calibration curve of each phenolic compound, appropriate volumes from each stock solution were diluted with methanol to obtain working solutions in the concentration range of 0.5-40mg/mL that were correlated with the measured area. The area of these EPAk was drawn and the corresponding concentration of phenolics was calculated based on the calibration curve. For each sample, the quantitative analyses were performed in triplicate at 290nm.

Reagents and standards. Ferulic, 3-hydroxy-4-methoxycinnamic, caffeic, p-coumaric, cinnamic, sinapic, 4- methoxycinnamic, 3,4- dihydroxybenzoic, 4-hydroxybenzoic and syringic acids were obtained from Sigma-Aldrich (Hamburg, Germany), gallic and vanillic acids were obtained from Fluka Chemie AG (Buchs, Switzerland). All reagents used were analytical grade, as well as formic acid (Vetec, Brazil) and methanol (TEDIA).

Tested samples. The antimicrobial activity of EPA and EPB was analysed at the Animal Microbiology and Immunology Laboratory of the "Universidade Federal do Vale do São Francisco" (Univasf), in Petrolina, Pernambuco State, using seventy-seven isolates of *Staphylococcus* spp. obtained from cases of subclinical bovine mastitis in dairy farms in the Northeast region of Brazil. They were also tested three multiresistant isolates, one being Methicillin-resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) and two from clinical strains of *S. aureus* ATCC, identified as ATCC 6538 and ATCC 25923, both methicillin-resistant.

Microdilution and minimum bactericidal concentration (MBC). The antimicrobial activity of the EPA and EPB was determined as the minimum bactericidal concentration (MBC) against *Staphylococcus* spp. isolates (n=77) and clinical strains of MRSA (n=3), *S. aureus* ATCC 6538 (n=1) and *S. aureus* ATCC 25923 (n=1). The samples plated on the TSA culture medium (Tryptone Soy Agar), were inoculated in tubes containing 3mL of Mueller Hinton (MH) broth medium, in order to perform microdilution according to Clinical And Laboratory Standards Institute (2006). After 24h, the medium was turbid at 0.5 on the Mac Farland scale (1x108 CFU/ml) and 0.1mL of this suspension was inoculated into tubes containing 9.9mL of MH broth (NCCLS 2002). Subsequently, microdilution was performed by placing 200µL of pure, sterile MH broth in each well of the microplate and then 200µL of each extract in the first well, followed by a 1:2 dilution and discarding the last 200µL, with the concentration varying from 0.26µg/ml to 550µg/ml. Then each well was inoculated with 10µL of the suspension containing the microorganisms. These wells and the positive control wells and negative control were incubated for 24h at 37°C. The contents of each of these wells were inoculated into petri dishes containing MH agar medium and incubated for 24h at 37°C. The lowest concentration of extract in which there was no growth of the microorganisms in the plaques was considered the MBC (NCCLS 2002). All samples were tested in triplicate.

Oxacillin susceptibility test. The sensitivity test was performed in all samples, in triplicate, by the modified Kirby-Bauer disk diffusion method (Clinical And Laboratory Standards Institute 2006), with microbial turbidity on the 0.5 scale of Mac Farland in MH broth. Samples were transferred with sterile swab to MH agar plates, in which the oxacillin-containing discs (1µg) were applied. The plates are incubated in an oven for 24h at 37°C. The breakpoint was determined according to (Clinical And Laboratory Standards Institute 2006).

Statistical analysis. The hypotheses are related to the antibacterial activity potential *in vitro* of two commercial extracts of propolis against bovine mastitis caused by *Staphylococcus* spp., in addition to relating them to resistance to oxacillin. The analyzes were performed observing the significance levels of the samples at 1% and 5%. The results were analyzed using analysis of variance

(ANOVA) and the probability p=0.05 was considered the critical value for all tests. The Tukey post-hoc test was used to separate statistically significant means. The SAS software Proc Gun model software was used for statistical analysis.

RESULTS

Analysis of the propolis extracts

The content of total phenolic and of total flavonoid varied between the samples. The EPA had a total phenolic content of 126.22mg (12.62%) and of total flavonoids of 51.06mg (5.10%) and the EPB of 73.12mg (7.31%) and 17.45mg (1.74%), respectively. The dry residue (soluble solids in methanol) content was 11.52% to the EPA and 10.37% to the EPB.

The components of each extract were identified by comparison with retention times of known chemical standards commonly found in propolis. The HPLC-DAD chromatograms of EPA and EPB indicated a similar profile of phenolic compounds in both extracts, with the presence, mainly, of cinnamic, ferulic, caffeic, coumaric, and 3,4-dihydroxybenzoic acids in all samples, which were detected according to their retention time and the UV spectral characteristics in comparison to those of standards.

The HLPC-DAD analysis revealed that the EPA presented a concentration of 226.55µg of coumaric acid, 222.55µg of cinnamic acid, 106.87µg of caffeic acid, 7.04µg of ferulic acid and 2.2µg of 3,4-hydroxybenzoic in 5mg of dry extract of propolis A, and for the EPB the concentrations were, respectively, 130.03µg, 130.03µg, 54.86µg, 3.99µg and 1.05µg in 5mg of dry extract of propolis B.

Antimicrobial activity of the propolis extracts

MBC against *Staphylococcus* spp. isolates ranged from 8.6µg/mL to 275µg/mL for the EPA, with a higher (P<0.01) number of sensitive isolates at the concentration of 68.7µg/mL (22/70, 31.4%), followed by the concentration of 34.3µg/mL (19/70, 27.1%). MBC ranged from 2.1µg/mL to 275µg/mL for the EPB, with a higher (P<0.01) number of sensitive isolates at the concentration of 137.5µg/mL (19/70, 56.2%), followed by concentration of 68.7µg/mL (19/64, 29.7%). These results indicated that EPA was more effective (P<0.01) in equal (22/70, 31.4%) or lower (33/70, 47.1%) concentration than 68.7µg/mL, while the EPB was more effective (P<0.01) in a higher concentration (38/64, 59.4%), presenting similar results at equal concentration (19/64, 29.7%) and lower result at concentrations below 68.7µg/mL (7/64, 10.9%) (Table 1). MBC against clinical strains of MRSA, ATCC 6538 and ATCC 25923 was also 68.7µg/ml for both EEP-A and EEP-B.

The analysis of the antimicrobial activity of the extract of propolis showed that 90.9% (70/77) of the 77 isolates of *Staphylococcus* spp. were susceptible to EPA, 83.1% (64/77) to EPB, 80.5% (62/77) susceptible to oxacillin and 19.5% (15/77) resistant to oxacillin. Among *Staphylococcus* spp. isolates susceptible to oxacillin 70.1% (44/62) were also susceptible to EPA and 80.6% (50/62) to EPB, whereas among those resistant to oxacillin 40.0% (6/15) was susceptible to EPA and 26.7% (4/15) to EPB (Fig.1).

Clinical strains of MRSA and *S. aureus* ATCC 6538 and *S. aureus* ATCC 25923 exhibited 100% susceptibility to both extracts.

DISCUSSION

This study brings important contributions to the scientific community due to the number of clinical strains of *Staphylococcus* spp. evaluated, as well as by the potential use of the propolis in the treatment of clinical cases of bovine mastitis and other infections caused by MRSA.

More than 300 constituents have been identified in different propolis samples (Marcucci 1995, Bankova et al. 2000, De Castro 2001, Park et al. 2002, Pietta et al. 2002, Alencar et al. 2007), which proportions depend upon of the place and time of collection (Park et al. 2002). Although the chemical composition and biological properties of propolis are variable, it generally has in its composition resin and vegetable balsam (50%), wax (30%), essential and aromatic oils (10%), pollen, as well as several other substances (5%) (Burdock 1998, Park et al. 2002, Pietta et al. 2002). The most important active compounds are flavonoids, terpenoids and phenylpropanoids (García-Lafuente et al. 2009), aromatic acids and phenolic compounds (Garza-González et al. 2010). The total phenolics and flavonoids contents may vary due to different factors, such as flora ecology (Park et al. 2002);

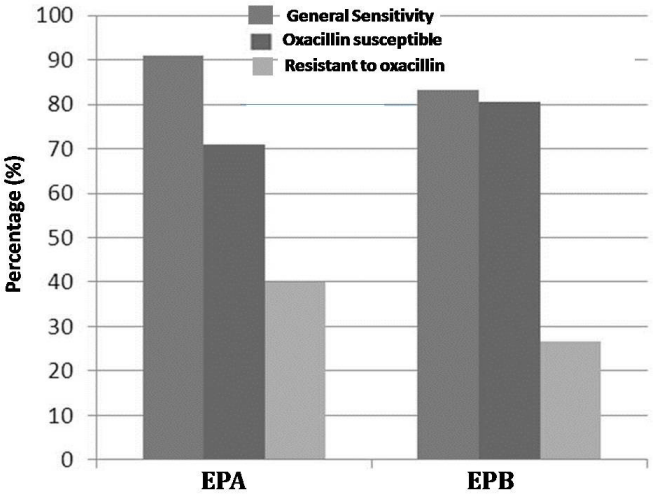


Fig.1. Antimicrobial activity of extract of propolis A (EPA) and B (EPB) on isolates of *Staphylococcus* spp. susceptible to several antibiotics and susceptible or resistant to oxacillin.

Table 1. Minimum bactericidal concentration (MBC) of the extracts of propolis against isolates of *Staphylococcus* spp.

	Total no./ sensitive	Minimum bactericidal concentration (µg/mL)											
		550	275	137.5	68.7	34.3	17.1	8.6	4.3	2.1	1.1	0.53	0.26
EPA	77/70	0^{d}	2^{d}	13^{c}	22^{a}	19^{b}	12^{c}	2^{d}	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
EPB	77/64	0^{e}	2^{d}	36^{a}	19^{b}	4^{c}	1^{e}	0^{e}	1^{e}	1^{e}	0^{e}	0^{e}	0^{e}

$^{a, b, c, d, e}$ Values are different (P<0.01).

resin collection period (Dos Santos et al. 2003); genetics of the queen bee (Park et al. 1998); local flora and collection region (Bankova 2005), among others. In spite of this, the values found in this paper were in accordance with the minimum limits set by the "Ministério de Agricultura, Pecuária e Abastecimento" (MAPA) of 5% for total phenolic and 0.5% for total flavonoids (Brasil 2001).

The obtained results confirmed that, the EPA and EPB contain considerable amounts of phenolic and flavonoids compounds, which were higher in EPA than EPB. In both extracts the total phenol content was higher than the total flavonoids content, a common finding for propolis from Brazil (Alencar et al. 2007, Moreira et al. 2008, Kalogeropoulos et al. 2009, Nunes et al. 2012, Coelho 2013), Greece, Cyprus (Kalogeropoulos et al. 2009), Czech Republic, Ireland and Germany (Al-Ani et al. 2018). The total phenol and flavonoids contents observed for EPA were similar to those of Kumazawa et al. (2004), El Sohaimy & Masry (2014) and Silva et al. (2015), lower than Alencar et al. (2007), Cabral et al. (2012), Peixoto et al. (2012) and El Sohaimy & Masry (2014), and higher than Tiveron et al. (2016). Cunha et al. (2004) observed a variation of 6.41 to 15.24% in the total phenol content, while Gonsales et al. (2006) verified variations between 0.05 and 0.63% in the contents of flavonoids and Sousa et al. (2007) verified a total flavonoid content of 0.06 to 0.38% for samples from São Paulo (Franca region) and from 0.12 to 2.11% for those from Minas Gerais (Passo region). This high variability in the total phenol and flavonoid content occurs due to the different sources of vegetable exudate, as well as by the location of the apiary (Park et al. 2002, Gonsales et al. 2006, Sousa et al. 2007). The soluble solids content in the both extract of propolis tested were higher than those observed by Sousa et al. (2007) and met the current legislation standards (Brasil 2001).

The HLPC-DAD analysis revealed mainly the presence of simple phenolic acids as caffeic, coumaric and cinnamic acids and methylated phenolic acids such as ferulic acid, and also 3,4-dihydroxybenzoic acid. These results agree with Al Naggar et al. (2016) that also found coumaric acid, ferulic acid and caffeic acid in Canadian propolis. Previous studies also have had describe presence of caffeic acid (Marcucci et al. 2001, Bankova et al. 2002, Kartal et al. 2002, 2003, Salomão et al. 2004, Ahn et al. 2007, Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Niculae et al. 2015, Oldoni et al. 2015, Tiveron et al. 2016, Al-Ani et al. 2018); coumaric acid (Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, Niculae et al. 2015, Oldoni et al. 2015, Tiveron et al. 2016, Afrouzan et al. 2018, Al-Ani et al. 2018); cinnamic acid (Salomão et al. 2004, Yang et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Tiveron et al. 2016, Al-Ani et al. 2018); and ferulic acid (Bankova et al. 2002, Kartal et al. 2002, Salomão et al. 2004, Ahn et al. 2007, Alencar et al. 2007, Mohammadzadeh et al. 2007, Barbarić et al. 2011, Coelho 2013, El Sohaimy & Masry 2014, Niculae et al. 2015, Oldoni et al. 2015, Bakdash et al. 2018).

Coumaric and cinnamic acids were the main components in EPA and EPB, contrasting with the results by previous reports that found ferulic acid (Alencar et al. 2007, Barbarić et al. 2011, El Sohaimy & Masry 2014, Niculae et al. 2015) caffeic acid (Kartal et al. 2002, Melliou & Chinou 2004, El Sohaimy & Masry 2014); p-coumaric acid (Jorge et al. 2008, Salomão et al. 2008, Afrouzan et al. 2018) and cinnamic acid (Katircioglu & Mercan 2006, Mohammadzadeh et al. 2007, Silva Filho et al. 2009) as the main component. Therefore, this was the first study to relate the joint action of cinnamic and coumaric acids to the antimicrobial activity of propolis, confirming that the propolis composition varies according to botanical and geographical origin (Bankova et al. 2002, Salomão et al. 2004, Bankova 2005, Popova et al. 2005, Sahinler & Kaftanoglu 2005, Barbarić et al. 2011, Huang et al. 2014, Shahbaz et al. 2015, Afrouzan et al. 2018, Bakdash et al. 2018).

The results showed that both EPA and EPB showed antimicrobial activity against isolates of *Staphylococcus* spp., in agreement with previous studies indicated that the antibacterial activity of propolis is more pronounced against Gram-positive bacteria (Langoni et al. 1996, Sforcin et al. 2000, Pinto et al. 2001, Fernandes Júnior et al. 2003, Auricchio et al. 2006, Trusheva et al. 2010, Aguiar et al. 2014). The antibacterial activity of the EPA, with 90.9% susceptibility, was similar to the Loguercio et al. (2006) and Coelho et al. (2010) which showed susceptibility of 94.4% in coagulase-positive *Staphylococcus*. When comparing the two extracts, it was verified that isolates of *Staphylococcus* spp. susceptible to common antibiotics, as well as those resistant to oxacillin, showed higher susceptibility to EPA, while isolates susceptible to oxacillin showed greater susceptibility to EPB. It is believed that the higher levels of phenolic compounds in EPA have been responsible for the greater susceptibility of isolates resistant to oxacillin, since phenolic compounds to bind to bacterial cell walls and prevent cell division and gr owth, as also observed in previous reports (Stapleton et al. 2004, El Sohaimy 2014, Al-Ani et al. 2018).

EPA demonstrated potent antibacterial activity against *Staphylococcus* spp. isolates in lower concentrations than EPB. These results agree with Kareem et al. (2015) who also observed this activity in Iranian propolis against *Staphylococcus* spp. MBC for EPA (34.3-68.6µg/mL) was similar to Cabral et al. (2009) and Rhajaoui et al. (2001); lower than Hayacibara et al. (2005), Alencar et al. (2007) and Tiveron et al. (2016) and higher than Santos et al. (2002) and Zeighampour et al. (2014). According to Cos et al. (2006), the ideal anti-infective concentration would be generally below 100µg/ml for extracts, confirming the excellent efficiency of the EPA in relation to EPB and to others studies.

Staphylococcus spp. have a high ability to develop mechanisms of antimicrobial resistance, which makes them a serious problem of global public health (Ratti & Sousa 2009, Garza-González et al. 2010, Reddy et al. 2017). This is the case with methicillin-resistant *Staphylococcus aureus* (MRSA) strains, which are resistant to all beta lactam antimicrobials (Adelman 2005), and often presents the multiple resistance phenomenon (Ratti & Sousa 2009). EPA and EPB inhibited significantly (100%) the clinical strains of MRSA and of *S. aureus* ATCC 6538 and ATCC 25923. Our results were similar to others reports in relation to the MRSA (Vera et al. 2011, Astani et al. 2013, Aguiar et al. 2014) and *S. aureus* ATCC 6538 (Hazem et al. 2017). In opposition, AL-Ani et al. (2018) observed a moderate anti-MRSA efficacy and Katircioglu & Mercan (2006) a low efficacy against *S. aureus* ATCC 25923, reinforcing the importance of the evaluation of multiresistant strains in the tests of antimicrobial activity of natural products (Cos et al. 2006). Low MBC, like observed in this study, might be advantageous in therapeutic level for appropriate treatment

of bacterial infections with regard to toxicity and stability of formulations (Astani et al. 2013).

The individual phenolic compounds present in the EPA and EPB were not identified and quantified, because submitting the entire extracts to antimicrobial activity studies seems to be more beneficial than to submit isolated constituents, since a bioactive individual component can change its properties in the presence of other compounds present in the extract (Borchers et al. 2004), corresponding to a synergistic effect.

The way propolis exerts its antimicrobial action is complex and occur, among other things, through inhibition of the bacteria growth by inhibiting of its enzymatic activity diminishing their effects on biological systems (Zeighampour et al. 2014); inhibition of cell division; collapse of the bacterial cytoplasm, cell membranes, and cell walls; bacteriolysis; and protein synthesis inhibition (Takaisi-Kikuni & Schilcher 1994 apud Fernandes Junior et al. 2005). *In vitro* studies have attributed the antimicrobial activity of propolis to the presence of phenolic compounds, flavonoids, phenolic acids and their esters (Pinto et al. 2001, Freitas et al. 2005, Katircioglu & Mercan 2006, Mohammadzadeh et al. 2007, Barbarić et al. 2011) or to the synergistic action of flavonoids and other active principles (Santos et al. 2002, Da Silva Filho et al. 2004, Barros et al. 2007, Sousa et al. 2007). Flavonoids (Uzel et al. 2005, Alencar et al. 2007, Cushnie & Lamb 2011) and phenylethyl ester of caffeic acid apEPAr to act by inhibiting bacterial RNA polymerase (Uzel et al. 2005), while phenolic compounds, such as caffeic acid, benzoic acid and cinnamic acid, cause functional and structural damages on the membrane or cell wall of microorganisms (Scazzocchio et al. 2005) or inhibition of bacterial replication (Rastogi et al. 2008) and galagin and caffeic acid cause enzymatic inhibition in bacteria (Koo et al. 2002). The results suggest that the antibacterial activity of EPA and EPB occurred mainly due to the content of phenolic compounds as previously described (Bankova et al. 1995, Marcucci et al. 2001, Popova et al. 2005, Estevinho et al. 2008, Ristivojević et al. 2016, Al-Ani et al. 2018), in particular by the content of cumaric and cinnamic acids (Popova et al. 2005, Sforcin 2007). However, it can not be ruled out that different substances may be involved in the antimicrobial activity, since Cabral et al. (2012) observed that G6 propolis, which had the lowest phenolic compound and flavonoid contents, showed the best antibacterial effect.

CONCLUSION

We could confirm that both extracts of propolis, whose main constituents are cumin and cinnamic acids, have high antimicrobial activity against the microorganisms studied, and EPA had also a high antimicrobial activity against oxacillin-resistant strains. These findings reinforce its potential use for the treatment of bovine mastitis.

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

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Hepatoprotective and antineoplastic potencial of red propolis produced by the bees *Apis mellifera* in the semiarid of Rio Grande do Norte, Brazil[1]

Jardel B. Silva[2]*, Kaliane A.R. Paiva[2], Kizzy M.F.M. Costa[2], Geysa A. Viana[2], Hélio N. Araújo Júnior[2], Lorena S. Bezerra[3], Carlos I.A. Freitas[2] and Jael S. Batista[2]

ABSTRACT.- Silva J.B., Paiva K.A.R., Costa K.M.F.M., Viana G.A., Araújo Júnior H.N., Bezerra L.S., Freitas C.I.A. & Batista J.S. 2019. **Hepatoprotective and antineoplastic potencial of red propolis produced by the bees *Apis mellifera* in the semiarid of Rio Grande do Norte, Brazil.** *Pesquisa Veterinária Brasileira 39(9):744-756.* Centro de Ciências Agrárias, Universidade Federal Rural do Semi-Árido, Av. Francisco Mota 572, Costa e Silva, Mossoró, RN 59625-900, Brazil. E-mail: jardelbezerra@bol.com.br

The objective of this study was to evaluate the hepatoprotective effect of the honey bee *Apis mellifera* ethanolic extract of the red propolis, obtained in four municipalities of the Rio Grande do Norte semi-arid region, through an *in vitro* evaluation of the antineoplastic potential in human hepatic carcinoma (HepG2) and normal cell lines (L929), and from the comet assay in hepatic cell lines (ZF-L hepatocytes) to evaluate the genoprotective potential of the extract. The hepatoprotective effect was also evaluated *in vivo* by the induction of chronic experimental hepatic lesions in rodents (*Rattus norvegicus* Berkenhout, 1769), Wistar line, by intraperitoneal administration of thioacetamide (TAA) at the dose of 0.2g/kg. The animals were distributed in the following experimental groups: G1 (control), G2 (treated with 500mg/kg ethanolic extract of propolis), G3 (treated with 500mg/kg of ethanolic extract and TAA) and G4 (treated with TAA). All rats were submitted to serum biochemical, macroscopic, histological and stereological biochemical exams of the liver. It was verified the genoprotective effect of red propolis since the mean damages promoted to DNA in cells tested with the extract were significantly lower than the mean of the positive control damage (hydrogen peroxide). The red propolis extract did not present cytotoxic activity to the tumor cells of human liver cancer, as well as to normal ones. The absence of cytotoxicity in normal cells may indicate safety in the use of the propolis extract. The results of the serum biochemical evaluation showed that the serum levels of the aminotransferase enzymes (AST) did not differ significantly between G1, G2 and G3 when compared to each other. G4 showed significant increase in levels compared to the other groups, indicating that the administration of the extract did not cause liver toxicity, as well as exerted hepatoprotective effect against the hepatic damage induced by TAA. The G3 and G4 animals developed cirrhosis, but in G3 the livers were characterized by the presence of small regenerative nodules and level with the surface of the organ, whereas in G4 the livers showed large regenerative nodules. The livers of the G1 and G2 animals presented normal histological appearance, whereas the livers of the G3 animals showed regenerative nodules surrounded by thin septa of connective tissue, and in G4 the regenerative nodules were surrounded by thick septa fibrous connective tissue. The analysis of the hepatic tissues by means of stereology showed that there was no statistical difference between the percentage of hepatocytes, sinusoids, and collagens in G1 and G2.

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[2] Centro de Ciências Agrárias, Universidade Federal Rural do Semi-Árido (UFERSA), Av. Francisco Mota 572, Costa e Silva, Mossoró, RN 59625-900, Brazil. *Corresponding author: jardelbezerra@bol.com.br

[3] Centro de Ciências da Saúde, Universidade de Fortaleza (Unifor), Av. Washington Soares 1321, Edson Queiroz, Fortaleza, CE 60811-905, Brazil.

In G3 the percentage of hepatocytes, sinusoids, and collagen did not differ significantly from the other groups. It was concluded that the ethanolic extract of the red propolis exerted a hepatoprotective effect, because it promoted *in vitro* reduction of the damage to the DNA of liver cells, antineoplastic activity in human hepatocellular carcinoma cell line (HepG2) and did not exert cytotoxic effect in normal cells or was able to reduce liver enzyme activity and the severity of cirrhosis induced by TAA *in vivo*.

INDEX TERMS: Hepatoprotection, antineoplastic potencial, red propolis, bees, *Apis mellifera*, Rio Grande do Norte, Brazil, northeast, cytotoxicity, genoprotection, rodents, rats.

RESUMO.- [**Potencial hepatoprotetor e antineoplásico da própolis vermelha produzida pela abelha *Apis mellifera* no semiárido do Rio Grande do Norte, Brasil.**] Este estudo objetivou avaliar o efeito hepatoprotetor do extrato etanólico da própolis vermelha da abelha *Apis mellifera*, obtido em quatro municípios do semiárido do Rio Grande do Norte, mediante avaliação *in vitro* do potencial antineoplásico em linhagens de células de carcinoma hepático humano (HepG2) e em linhagens de células normais (L929), além do ensaio cometa em linhagens de células hepáticas (hepatócitos ZF-L) para avaliar o potencial genoprotetor do extrato. O efeito hepatoprotetor também foi avaliado *in vivo* através da indução de lesões hepática experimental crônica em roedores da espécie *Rattus norvegicus* (Berkenhout, 1769), linhagem Wistar, pela administração intraperitoneal de tioacetamida (TAA) na dose de 0,2g/kg. Os animais foram distribuídos nos seguintes grupos experimentais: G1 (controle), G2 (tratados com 500mg/kg de extrato etanólico da própolis), G3 (tratados com 500mg/kg de extrato etanólico e TAA) e G4 (tratados com TAA). Todos os ratos foram submetidos aos exames bioquímico sérico, anatomopatológico macroscópico, histológico e esteriológico do fígado. Foi constatado o efeito genoprotetor da própolis vermelha uma vez que as médias dos danos promovidos ao DNA em células testadas com o extrato foram significativamente inferiores à média dos danos do controle positivo (peróxido de hidrogênio). O extrato da própolis vermelha não apresentou atividade citotóxica para células tumorais de câncer de fígado humano, bem como para normais. A ausência de citotoxicidade em células normais, tal como constatado, pode indicar segurança no uso do extrato da própolis. Os resultados da avaliação bioquímica sérica demonstraram que os níveis séricos das enzimas aminotransferase (AST) não diferiram significativamente entre G1, G2 e G3, quando comparadas entre si. No G4 houve aumento significativo dos níveis em relação aos demais grupos, indicando que a administração do extrato não causou toxicidade hepática, bem como exerceu efeito hepatoprotetor frente ao dano hepático induzido pela TAA. Os animais dos G3 e G4 desenvolveram cirrose, porém no G3 os fígados caracterizaram-se pela presença de pequenos nódulos regenerativos e nivelados com a superfície do órgão, enquanto que no G4 os fígados apresentaram grandes nódulos regenerativos. Os fígados dos animais G1 e G2 apresentaram aspecto histológico normal, enquanto que os fígados dos animais do G3 apresentaram nódulos regenerativos circundados por finos septos de tecido conjuntivo, e nos do G4 os nódulos regenerativos foram circundados por espessos septos de tecido conjuntivo fibroso. A análise dos tecidos hepáticos por meio de estereologia mostrou que não houve diferença estatística entre o percentual de hepatócitos, sinusoides e colágenos nos G1 e G2. No G3 o percentual de hepatócitos, sinusoides e colágeno não diferiu significativamente dos demais grupos. Concluiu-se que o extrato etanólico da própolis vermelha exerceu efeito genoprotetor, por promover *in vitro* redução do dano ao DNA de células hepáticas, atividade antineoplásica em linhagem celular de carcinoma hepatocelular humano (HepG2) e não exerceu efeito citotóxico em células normais ou efeito hepatoprotetor *in vivo* com diminuição da gravidade da cirrose induzida por TAA.

TERMOS DE INDEXAÇÃO: Hepatoproteção, antineoplásicos, própolis vermelha, abelhas, *Apis mellifera*, Rio Grande do Norte, Brasil, nordeste, citotoxicidade, genoproteção, ratos.

INTRODUCTION

Propolis is composed of resinous and balsamic material collected by bees on tree branches, flowers, pollen, buds, and exudates. It is a complex mixture in which bees add salivary secretions in the hive. The resin is used by bees to protect the hive against the proliferation of microorganisms, and to maintain internal temperature, repairing honeycombs, embalming insects, and closing holes and cracks (Silva et al. 2006, Zilse & Silva 2012).

Depending on its color, odor, and consistency, the characteristics of propolis are associated with its region of origin, botanical source, and chemical composition. Thus, propolis can range from green, red to brown, black, and yellow (Bankova 2005, Lopes 2014). Red propolis is in the newest, being the 13th type of cataloged propolis, which revealed a chemical composition rich in isoflavonoids. Its botanical origin is from the *Dalbergia ecastophyllum* plant, a legume rich in flavonoids. This species is reported to be from Cuba and Venezuela, originating from the plants *Clusia nemorosa* and *Clusia scrobiculata*, *Clusiaceae* family (Li et al. 2008, Bueno-Silva et al. 2013).

Brazil is the world's second-largest producer of propolis, exporting about 160 tons annually, being only after China. Besides being good for health, the consumption of organic products encourages rural producers to maintain good agricultural practices for environmental preservation, using soil, water, air and other natural resources responsibly (Lima 2006, Oldoni et al. 2015).

The literature often shows the biological properties of propolis related to the presence of a variety of biologically active compounds, especially the phenolic compounds, which include flavonoids with outstanding antioxidant and anti-inflammatory activities and components (Al Naggar et al. 2016). Different research has shown that the significant amounts of antioxidants in the diet contribute to the prevention of serious, chronic and inflammatory diseases, which are associated with the formation of radicals during the oxidation process, since natural antioxidants can decrease oxidative

stress and consequently promote oxidative stress protection against DNA damage (Carratú & Sanzini 2005, Horst & Lajolo 2012, Teerasripreecha et al. 2012, Daleprane & Abdalla 2013).

As the liver is the main organ of metabolism of compounds, many liver diseases appear by its exposure to toxic substances, and the oxidative stress promoted by these substances is very important in the pathophysiological mechanisms of these diseases (Voican et al. 2011). As the propolis has bioactive substances with potential antioxidant, anti-inflammatory, and genoprotective, the red propolis ethanolic extract may prevent or minimize damage to liver cells when exposed to hepatotoxic substances. Thus, the objective of this study was to evaluate the hepatoprotective potential of the *Apis mellifera* red propolis ethanolic extract obtained in the semiarid region of Rio Grande do Norte, Brazil, through the chronic experimental induction of liver damage in rodents (*Rattus norvegicus* Berkenhout, 1769), Wistar strain, using thioacetamide, the *in vitro* evaluation of the antineoplastic effect of human liver carcinoma (HepG2) cell line extract, and the genoprotective potential in liver cell lines (hepatocytes ZF-L).

MATERIALS AND METHODS

Red propolis extract

Acquisition and elaboration of propolis extract. Four samples of red propolis produced by the bee *Apis mellifera* from hives installed in the municipalities of Parnamirim (samples A and B) and Mossoró (samples C and D) were collected from February to March 2018. The preparation of the extracts used samples of 200g of propolis, subdivided into small portions, and submitted to drying in an air circulation oven at 40°C for 48h, and then crushed in a domestic blender. Cold maceration was performed at a ratio of 1:6.25 (m/v) for each 1g of crushed propolis, using 6.25mL of 70°GL cereal alcohol. The samples were placed in properly identified amber glass and shaken manually for 30 seconds daily for 20 consecutive days of infusion, according to Garcia et al. (2004a). In the next step, the filtering of the supernatant was performed on filter paper and later on cotton, to retain all the insoluble particles. The filtration was refrigerated in a freezer (-20°C) for 45 minutes to remove the wax and then another supernatant filtration was done, followed by centrifugation, obtaining the ethanolic extracts of the propolis.

Estimation of total phenol content. The Folin Ciocalteu method (Singleton et al. 1999) was used to determine phenolic content. Each propolis extract sample (5mL) was diluted into 50mL with distilled water. This solution (0.5mL) was mixed with 2.5mL of 0.2N Folin Ciocalteu reagent for 5 minutes and 2mL of 75g/L sodium carbonate (Na_2CO_3).

After 2 hours of incubation at room temperature, the absorbance of the reaction mixture was measured at 760nm against a methanol blank in a spectrophotometer (Series 2000, CECIL Instruments, Cambridge, England). Gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) (0-200mg/L) was the standard used to obtain the calibration curve. The mean of three readings was used, and the total phenolic content was expressed in mg of gallic acid equivalents (GAE)/100g of propolis.

Estimation of total flavonoid content. Total flavonoid content was determined using the Dowd Method adapted by Grand et al. (1994). Thus, there were 5mL of 2% aluminum chloride (AlCl3) (Labosi, Paris, France) mixed in methanol (Fluka Chemie, Switerland) with the same volume as a propolis extract solution (0.01mg/mL). Then, a blank solution of a 5mL propolis extract solution was added to 5mL methanol without AlCl3, and then absorbance readings at 415nm were taken on a spectrophotometer (Series 2000, CECIL CE 2041, Cambridge, England). Total flavonoid content was determined using a standard quercetin curve (Sigma-Aldrich Chemie, Steinheim, Germany) (0-50mg/L). The mean of three readings was used and expressed as mg quercetin equivalents (QE)/100g of propolis.

Antioxidant activity by the DPPH method. For the assessment of the antioxidant activity, the *in vitro* photocolorimetric method of free radical DPPH (2.2-diphenyl-1-picrylhydrazine) was used, described by Mensor et al. (2001). The extracts were diluted in methanol at different concentrations (100, 50, 40, 20, 5, and 2ppm). Then, 1mL of each concentration was placed in test tubes along with 1mL of a 60µmol/L DPPH methanolic solution. After 30 minutes, the absorbance of the samples was measured by spectrophotometer at a wavelength of 520nm, with only methanol as blank.

The inhibition percentage for each extract concentration was obtained from the absorbance ratio with the absorbance of a solution containing 1mL methanol and 1mL DPPH solution. All analyses were triplicated. After the free radical percentage inhibition values, they were analyzed in Origin 7.0 Software to obtain a graph showing values, calculating the 50% inhibitory concentration of the DPPH radical (IC50).

Genoprotective potential assessment. Sample A of the red propolis extract was used to assess the genoprotective effect since it presented better results in terms of total phenols, total flavonoids, and antioxidant activity. The comet assay assessed the genoprotective effect in liver cell line (hepatocytes ZF-L) ($0.7x10^5$ cells/mL) cultured in DMUL (Dulbecco modification of Minimum Essential Media; GIBCO®) supplied with 10% fetal cattle serum and 1% antibiotics. Liver cells were exposed to hydrogen peroxide (H_2O_2; 150µM for 2 hours exposure) for genotoxicity induction (positive control) and sterile distilled water (negative control). Cell cultures were also co-treated with increasing concentrations of propolis (100, 250 and 500µg/mL) and H_2O_2 (150µM) for 2 hours at 37°C in a 5% CO_2 atmosphere (test group).

The cells were homogenized in 0.8% agarose and spread on prepared slides and immersed in lysis solution for 1 hour, followed by neutralization. Subsequently, the slides were kept in electrophoresis buffer at 4°C for 20 minutes, followed by running for 20 minutes, $1.6Vcm^{-1}$. The slides were stained in an ethidium bromide solution (20µg/mL) and analyzed using a fluorescence microscope. The degree of DNA damage was visually identified by analyzing the tail formed by the DNA fragments, and the tail size was proportional to the extent of the damage caused (Mezzalira et al. 2014). One hundred comets per slide were analyzed and classified by visual analysis into five categories (0, 1, 2, 3 and 4), representing the percentage of DNA in the comet's tail, showing the degree of damage in the cell (Lovell et al. 1999).

The damage index (DI) was obtained by the following formula:

$$DI = \sum_{i=0}^{4} n_i \times i$$

In which n_i is the number of cells with damage level i (0, 1, 2, 3, or 4).

The protective effect of test samples on H_2O_2-induced genotoxicity (150µM for 2 hours exposure) was calculated according to Waters et al. (1990), and the formula: % reduction = (A-B/A-C)x100, in which A corresponds to the DI induced by H_2O_2, B corresponds to the DI induced by genoprotective treatment (H_2O_2 + test sample), and C corresponds to the DI for the negative control (distilled water).

Statistical analysis. The ANOVA and Newman-Keuls tests were used in the Prism® version 5.0 software (GraphPad Prism Software)

for the statistical analysis of the genoprotective effect, and the results were considered significant when $p<0.05$.

Antineoplastic effect. The red propolis extract sample A was used to assess the antineoplastic activity through the cytotoxicity assay in a human hepatocellular carcinoma cell line (HepG2). For the comparison and evaluation of the selective effect of cytotoxicity, a healthy cell line (fibroblasts-L929) was used.

The evaluation of the cytotoxic activity of the red propolis ethanolic extract in neoplastic (HepG2) and normal cells (fibroblasts-L929) was performed by the MTT (3-bromide (4.5-dimethyl2 thiazolyl)-2.5-diphenyl-2H tetrazolium) according to the methodology described by Mosmann (1983). The cells were added in a 96-well plate ($1.5x10^5$ cells/well), supplied with 10% fetal bovine serum and treated with red propolis extract at concentrations of 500, 250, 125, 62.5, 31.2, 15.6, 7.81, 3.90, 1.95, and 0.97µg/mL. Subsequently, they were incubated for 24h in a 5% CO_2 greenhouse at 37°C, and then 25µL of the MTT solution was added, and the absorbance was read on a 595nm spectrophotometer.

Data analysis. The mean absorbance data from the samples were compared by analysis of variance ANOVA followed by Tukey post-test using the Prisma version 5.0 program (GraphPad Software), with a significance level of $p<0.05$. The percentage of inhibition xlog of concentration and its IC_{50} and respective confidence intervals (95%) from nonlinear regression were also recorded using the Prisma version 5.0 program (GraphPad Software).

***In vivo* hepatoprotective activity**

Experimental animals and bioethics. Forty male adult rodents (*Rattus norvegicus* Berkenhout, 1769), Wistar strain, 60-day-old, weight of 200g were used, provided by the Rio Grande do Norte State University (UERN) vivarium. The animals were housed in propylpropylene cages, kept at temperature (21°C ± 2°C) and controlled humidity (55% ± 10%) with 12 hours light/dark photoperiod, fed with commercial food (Purina®) and *ad libitum* water throughout the experimental procedure. According to the manufacturer (Purina®), the food composition was approximately 23% of crude protein, 4% of total lipid, 5% of fiber and 12% of minerals.

The experimental protocols used in this study were submitted to the approval of the "Ethics Committee on Animal Use" of the "Rio Grande do Norte State University" (CEUA-UERN), with protocol number 002/2018.

Experimental design of liver cirrhosis. The animals were divided into four experimental groups and the serum biochemical parameters indicating liver damage, macroscopic and histopathological liver parameters were evaluated.

Group I (Control): It had ten animals that received via oral only water daily for 14 weeks.

Group II: It had ten animals that received via oral 500mg/kg propolis red ethanolic extract daily for 14 weeks.

Group III: It had ten animals that received via oral 500mg/kg propolis red ethanolic extract daily for 14 weeks and 0.2g/kg intraperitoneal thioacetamide (TAA) three times a week during the same period.

Group IV: It had ten animals that received intraperitoneal 0.2g/kg TAA three times a week for 14 weeks.

The sample of red propolis extract A was used to evaluate the *in vivo* hepatoprotective effect. The route of administration and the dose of TAA used to induce liver cirrhosis were established as described by Amin et al. (2012). The choice of administration routes and propolis extract doses were also based on previous studies in the literature and established as described by Mahmoud et al. (2015).

Blood samples collection and processing. The animals were anesthetized using ketamine hydrochloride solution (100mg/kg) and xylazine hydrochloride (100mg/kg) by intraperitoneal route. Then, a laparotomy was performed through an incision over the alba line to allow access to the hepatic vein. From each animal, 3mL of blood was collected using a 30x0.7 syringe and needle, placed in tubes without the addition of anticoagulant and then tilted and kept at rest to obtain blood serum. The serum obtained was stored in a freezer (-80°C) for further biochemical analysis.

Biochemical analysis of liver function. Blood serum samples were analyzed to determine the following liver damage markers: aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), total protein (TP), glucose (GLUC), triglycerides (TRIG) and total bilirubin (BILT). The samples were tested by commercial reagents (LABTEST, Belo Horizonte/MG) and then read in a biochemical analyzer by spectrophotometry (BIOPLUS 2000).

Pathological examination. Immediately after the blood collection, the animals were euthanized by cervical dislocation under deep anesthesia. Then, the animals were individually weighed with precision scales and submitted to necropsy, using the technique proposed by Vasconcelos (1996), with an external examination of the animal, followed by the opening of the thoracic, abdominal and cranial cavities and the macroscopic examination of the organs. Each animal's liver was weighed and examined to identify possible changes from the normal pattern, especially considering changes in weight, size, color, and consistency, as well as the presence of nodules.

Analysis of the results. The mean ± standard deviation was used to express the data obtained from serum biochemical parameters and liver weight. To evaluate possible differences between treatments, the data obtained were submitted to one-way ANOVA analysis of variance, followed by Tukey's multiple comparison post-test, considering $p<0.05$ as significant. Data were analyzed using the SAS statistical program (2002).

Histopathological examination. Organ fragments were fixed in 10% formaldehyde buffered solution and routinely processed for histology, embedded in paraffin, cut to 5µm in thickness and stained with hematoxylin-eosin (HE). Liver tissue specimens were also subjected to Gomori trichrome staining to identify collagen fibers, as established by Andrade et al. (1999).

Stereological assessment. The livers were sectioned following a uniform and systematic random sampling pattern, as described by Marcos et al. (2012). In two liver fragments stained by the hematoxylin-eosin and Gomori trichrome method, five microscopic fields were randomly evaluated per animal and captured on the 20x objective using a video microscope system (Leica). A total of 20 blindly microscopic fields for each animal were analyzed. A test system composed of 50 test points (PT) was superimposed over the image of each field using the Pro-Plus Image software (Version 4.5.0.29.), evaluating hepatocytes, sinusoids and collagen fibers. The volumetric density (Vv) of these parameters was evaluated by the following formula: Vv = PP/PT (%) (PP represents the points reaching the structure).

The values were expressed as mean ± standard deviation, and the minimum and maximum values were expressed using the SAS version 8.0 statistical program. After analyzing the parametric assumptions, statistical differences between the different experimental groups were obtained by analysis of variance (One-Way ANOVA), followed by Tukey's test. Percentage of data underwent sine arc transformation $\sqrt{x}$.

RESULTS AND DISCUSSION

Phenols and total flavonoids contents

The phenol and total flavonoid contents ranged from 74.92±0.51 to 141.07±1.27mg GAE/100g and 2.42±0.17 to 8.35±0.28mg QE/100g, respectively (Table 1). The extracts made with red propolis from Parnamirim had higher levels of phenols and total flavonoids, observing that the levels vary according to the location of the propolis. Thus, this data is similar to those data published in the literature, which stated that the phenol and total flavonoid contents in a propolis sample are closely related to the flora ecology of each region visited by the bees (Cardinault et al. 2012, Nunes & Guerreiro 2012). Other studies also showed that the red propolis collected in Alagoas (Frozza et al. 2013), Cuba (Piccinelli et al. 2005) and China (Hatano et al. 2012) had a varied chemical composition and contained significant amounts of phenols and total flavonoids. Several studies highlight that many biological functions of the propolis can be attributed to its antioxidant and anti-inflammatory components, promoted by phenolic compounds (Freitas et al. 2008, Lustosa et al. 2008, Araújo et al. 2016). Thus, the finding of high levels of phenols and total flavonoids are indicators of the quality of red propolis from Rio Grande do Norte.

Antioxidant activity

The ethanolic extract of red propolis was submitted to the antioxidant activity test, using the DPPH radical sequestration methodology. As the antioxidant activity is the ability to eliminate reactive oxygen/free radical species by hydrogen donation, the DPPH reduction test is used as a stable free radical to verify the antioxidant activity of different propolis extracts of different bees (Ahn et al. 2007). The sequestering capacity of the DPPH radical is represented by the IC_{50} values (%) of the samples compared to pure quercetin, used as a reference standard. Thus, the lower the IC_{50} value (%), the greater the sequestering capacity of the radical and the higher the antioxidant activity.

The antioxidant activity ranged from 141.07±0.21µg/mL to 54.14±1.50µg/mL for propolis samples collected from Mossoró and Parnamirim, respectively (Table 1). Melo et al. (2010) classified the antioxidant activity of an extract as good (IC_{50} <65µg/mL), moderate (IC50 <152µg/mL) and low activity (IC50 >152µg/mL). This classification showed that in general, the red propolis of this study had an antioxidant activity that ranged from good (samples A and B) to moderate (samples C and D). In a study by Frozza et al. (2013), the red propolis collected in Sergipe had an IC_{50} of 270µg/mL. Similar results were obtained by Pinheiro (2009) with red propolis from the same geographical region, with an IC_{50} of 294µg/mL. Thus, the red propolis samples of this study had expressive IC_{50} (%), when compared with these authors.

After finding a high antioxidant activity of red propolis from Alagoas, Aguiar et al. (2018) suggested that it can be used to help in the treatment of degenerative diseases, including Alzheimer's. From the point of view of chemical composition and antioxidant activity, the results in this study are very promising, and the red propolis of Rio Grande do Norte can be a viable alternative economically and with pharmacological efficacy.

Genoprotective effect

Considering that DNA damage can interfere with essential cellular processes such as replication and transcription and can lead to cell death and induce cancer-causing mutations that contribute to the aging process, it is important to evaluate possible events that may lead to genomic instability as well as those linked to maintaining genome stability (Hoeijmakers 2009). Genotoxicity studies play an important role in the development of new drugs and should be carried out in the early stages of their development to identify potential genotoxic and/or carcinogenic activity and to assist in obtaining new, less toxic chemical structures. Thus, genotoxicity tests are used as a scan of the toxicological spectrum of natural or synthetic substances capable of producing genotoxic effects and assisting in the discovery of genoprotective substances for the everyday use of DNA mutation prevention (Aquino 2010, Niwa et al. 2013).

The comet assay is used to detect DNA damage. In this assay, the genetic material of the cell is fragmented using chemicals or radiation and then submitted to an electrophoresis process, so the fragmented DNA migrates over the agarose gel and the broken ends of the DNA molecule of negative charge become free to migrate in the electric field towards the anion, forming a structure similar to a comet. The length and amount of DNA in the comet's tail reflect damage to the genetic material (Fikrová et al. 2011).

Thus, the results when using the red propolis ethanolic extract demonstrated a genoprotective effect since when the means of the cell DNA damage indices (hepatocytes ZF-L) were compared in all the tested concentrations of the extract, no significant differences were observed in the negative control (sterile distilled water) (Table 2).

It was also found that the means of DNA damage at all tested concentrations of the red propolis ethanolic extract was significantly lower than the positive control (H2O2) mean, which is often used as a standard genotoxic substance in *vitro* exposure, enable to promote DNA fragmentation.

Table 1. Mean ± standard deviation of phenol contents, total flavonoids and antioxidant activity of four samples of *Apis mellifera* red propolis obtained from the semiarid of Rio Grande do Norte, Brazil

Sample	Geographic origin	Total phenols (mg GAE/100g ± SD)	Total flavonoids (mg QE/100g ± SD)	RSA IC_{50} (µg/mL ± SD)
A	Parnamirim/RN	141.07 ± 1.27	8.35 ± 0.28	61.04 ± 0.31
B	Parnamirim/RN	120.41 ± 1.13	6.12 ± 0.30	54.14 ± 1.50
C	Mossoró/RN	74.92 ± 0.51	2.42 ± 0.17	141.07 ± 0.21
D	Mossoró/RN	86.09 ± 0.47	3.60 ± 0.40	129.10 ± 0.49

GAE = equivalent gallic acid, IC_{50} = inhibitory concentration of 50%, QE= quercetin equivalent, RSA (CI_{50}) = radical sequestration activity, SD = Standard deviation.

The percentages of genotoxicity reduction were 90.17±3.01, 71.15±2.64 and 43.07±4.99, respectively, for the extract concentrations of 500, 250 and 100µl/mL (Table 3), proving the genoprotective effect of ethanolic extract of red propolis.

The literature often highlights that the biological properties of propolis are related to the presence of a variety of biologically active compounds, mainly phenolic compounds, which include flavonoids (Al Naggar et al. 2016). This finding fits in the red propolis analyzed in this study since in the evaluation of the chemical composition of the extract, high levels of phenolic compounds and flavonoids, and high antioxidant activity was found. Phenolic compounds have been reported to have genoprotective activity because of their antioxidant activity (Gontijo et al. 2014). Thus, the antioxidant activity of red propolis could be the possible explanation for genoprotective effect. The bioactive substances with antioxidant capacity in the extracted sample analyzed probably interacted with H_2O_2, blocking its deleterious effect on DNA, and exerting genoprotective activity. There are no studies in the literature evaluating the genoprotective effect of red propolis using a comet assay, highlighting the novelty of this study. The finding of the genoprotective effect on ZF-L hepatocyte lineage suggested that the red propolis obtained in Rio Grande do Norte may be able to assist in the prevention of diseases triggered by DNA damage.

Antineoplastic activity assessment

Although the studies and investments held in research, cancer chemotherapy still discourages scholars due to multiple drug resistance and serious side effects from morphological and physiological similarities between normal and transformed cells (Kamb 2005). Therefore, it is of great importance to identify natural molecules with potential therapeutic activity for future clinical studies and as a source of knowledge for the synthesis of new compounds with more effective and less toxic antitumor activity (Sawicka et al. 2012).

In vitro cytotoxicity tests are part of the initial screening to determine the possible antitumor potential of a natural product (Oliveira et al. 2012). The antineoplastic activity of the ethanolic extract of the red propolis of Rio Grande do Norte was evaluated by the human hepatocellular carcinoma cell line (HepG2) cytotoxicity assay, by the MTT test developed by Mosmann (1983) to estimate proliferation and cell survival. The literature defined the assay as appropriate to estimate cytotoxicity quickly and accurately, and it is considered one of the most widely used tests by the National Cancer Institute (NCI) for its use in antineoplastic activity screening programs for anticancer drug screening (Oliveira et al. 2012). The test is based on the ability of succinate dehydrogenase, a Krebs cycle enzyme active in viable cell mitochondria, to convert the soluble and yellowish soluble water tetrazolium salt (MTT) into purple color formazan crystals. Thus, the amount of formazan measured by spectrophotometry is directly proportional to the number of viable cells (Porto et al. 2011).

Several *in vitro* and *in vivo* studies have demonstrated the antiproliferative potential of Brazilian propolis and its isolated constituents, and some results are favorable for its use (Miguel & Antunes 2011, Salatino et al. 2011, Sforcin & Bankova 2011, Cardinault et al. 2012). One of these studies is from Li et al. (2008), who showed that red propolis from northeastern Brazil exhibited antitumor potential, related to the presence of flavonoids and Pterocarpus. In the compounds evaluated, flavanone 7-hydroxy-6-methoxiflavanone and isoflavone mucronulatol showed a potent cytotoxic action against the lines of melanoma (B16-BL6), Lewis lung carcinoma (LLC), human pulmonary adenocarcinoma (A549), metastatic fibrosarcoma (HT-1080) and LLC, A549, respectively. In flavanones, the absence or presence of hydroxyl on carbon 3 (C-3) may be involved in the cytotoxic potential of these compounds. Regarding isoflavones, the increase in the number of methoxyl groups in the base structure necessarily increased the cytotoxic potential.

Also, Awale et al. (2008) evaluated the cytotoxic activity of methanolic extract of red propolis and some of its isolated compounds revealing that both the total extract and a pterocarpane (6aR, 11aR) -3,8-dihydroxy-9-methoxypterocarpane) showed high cytotoxicity against human pancreatic cancer cells (PANC-1). The mechanism of death is associated with a non-apoptotic time-dependent process that does not lead to deoxyribonucleic acid (DNA) fragmentation but necrotic morphological changes. The work by Frozza (2016) is also highlighted for finding a cytotoxic effect of the ethanol extract of red propolis from the states of Sergipe and Alagoas, against tumor cells of the Hep-2 strain. Based on this result, the authors concluded that red propolis became a promising candidate

Table 2. Mean ± standard deviation of DNA damage indexes (idDNA), analyzed by comet assay for the assessment of the genoprotective effect of *Apis mellifera* red propolis ethanolic extract after liver cell exposure (hepatocytes ZF-L), for 2h in three different concentrations of the extract, hydrogen peroxide (H_2O_2) and sterile distilled water

Parameter	The concentration of ethanolic extract of red propolis (µl/mL)			Control +	Control -
	500	250	100		
IdDNA	8.15 ± 2.05a	7.99 ± 2.32a	6.97 ±2.42a	158.38±13.25b	7.80±2.32a

a,b = Means followed by different lowercase letters on the line means statistical difference ($p<0.05$) and the means of the same line followed by equal letters are statistically equal, positive control (+) = H_2O_2, negative control (-) = distilled water.

Table 3. Mean ± standard deviation of the percentage reduction in genotoxicity analyzed by the comet assay for the evaluation of the genoprotective effect of the *Apis mellifera* red propolis ethanolic extract in ZF-L hepatocyte lineage, co-treated with three concentrations of propolis and hydrogen peroxide samples (H_2O_2)

Parameter analyzed	The concentration of ethanolic extract of red propolis (µl/mL)		
	500	250	100
% genotoxicity reduction	90.17 ± 3.01	71.15 ± 2.64	43.07 ± 4.99

to inhibit cell growth and contributed to the different steps related to the carcinogenesis process.

As an antitumor activity parameter, the NCI in its anticancer drug screening program considers compounds with antineoplastic activity when they have IC_{50} <30μg/mL (Itharat et al. 2004). The IC_{50} index = cytotoxicity index corresponding to the concentration reducing the number of cells to 50%, the ethanolic extract of Rio Grande do Norte red propolis presented IC_{50}>500μg/mL for the normal cell line (L929) and 358μg/mL for human liver cancer (HepG2) tumor cells. Doxorubicin as a reference drug showed cytotoxicity in all cell lines tested, with IC_{50} values ranging from 0.4818μg/mL to 2.343μg/mL for L929 and HepG2 strains, respectively (Table 4). Red propolis was not toxic to normal cells, even at the highest concentration tested (500μg/mL). This fact may indicate safety for the use of the red propolis ethanolic extract.

Serum biochemistry

The evaluation of liver function in rodents (*Rattus norvegicus* Berkenhout, 1769), Wistar strain, in the different experimental groups in this study was the determination of serum biochemical parameters, which demonstrated that the mean values of AST enzyme were within normal limits for the species and did not differ significantly between G1, G2, and G3 when compared to each other. However, there was a significant increase in levels in G4 compared to the other groups. Regarding ALT serum levels, there was no significant difference between G1 and G2, and there was a significant increase in the mean values G3 and G4 than the other groups (Table 5).

The liver produces more than 60 transaminases, two of which are of major clinical importance, AST and ALT (Kwo et al. 2017). Their levels may cause liver damage because when liver cells are damaged or destroyed, these enzymes are released into the bloodstream, being sensitive indicators of liver damage (Hasan et al. 2013). Thus, the analysis of serum biochemical parameters confirmed the deleterious effect of TAA, considering that the mean values of these enzymes were significantly higher than the values in the other groups. The results obtained in the evaluation of the enzyme AST showed that its levels did not change in G1, G2, and G3, indicating that the administration of the red propolis ethanolic extract did not cause liver toxicity, and exerted hepatoprotective effect against the TAA-induced liver damage.

The hepatoprotective effect of red propolis obtained in Cuba was evaluated by Remirez et al. (1997) by induction of liver toxicity by the administration of 64mg of ethyl alcohol in mice, pretreated at the doses of 25, 50 and 100mg of ethanolic extract of propolis. In the study, the authors found that in the propolis-treated group, there was a significant reduction in serum ALT levels and that the hepatoprotective effects of propolis were dose-dependent. The authors suggested that red propolis exerted a hepatoprotective effect due to its antioxidant properties, which exerted free radical scavenging effects, capable of promoting deleterious effects on the liver. Harrizul et al. (2018) also observed a reduction in serum enzyme indicating ALT liver damage, and a reduction in liver cell degeneration scores and inflammatory reaction in pre-treated rodents with 280mg/kg of propolis obtained in Indonesia, which were experimentally submitted to hepatic damage induced by hepatotoxic drug valproic acid at a dose of 350mg.

Regarding the mean values of serum albumin and total protein levels, the values were also within the normal range for the species, and no significant difference was observed between G1, G2, and G3. In G4 there was a significant reduction in values (Table 5). Measurements of albumin and total proteins are also markers often used as a test of liver function since most plasma proteins are produced exclusively by the liver (Schinoni 2006). Thus, this result could also indicate

Table 4. Inhibitory concentration values (IC_{50}) promoted by the ethanolic extract of *Apis mellifera* red propolis and doxorubicin chemotherapy against cell lines L929 and HepG2 by MTT after 72h exposure

Compounds	CI_{50} Cell lines μG/mL*	
	L929	HepG2
Hydroethanolic extract of red propolis	>500 μg/mL	358 μg/mL
Doxorubicin	0.4818 μg/mL	2.343 μg/mL

*Values of three independent experiments and presented in IC_{50} values obtained by nonlinear regression with 95% confidence interval.

Table 5. Mean ± standard deviation values of serum biochemical parameters in rodents (*Rattus norvegicus* Berkenhout, 1769), Wistar strain in experimental groups G1 (control), G2 (treated with ethanolic extract of red propolis), G3 (treated with ethanolic extract of propolis TA4), G4 (TAA-treated) and reference values

Parameters	Experimental groups				Reference
	G1	G2	G3	G4	
AST (U/L)	97.14 ± 4.77a	108.33 ± 8.38a	114.22 ± 7.31a	184.7 ± 19.87b	131.33 ± 43.98
ALT (U/L)	62.16 ± 20.21a	67.99 ± 11.72a	114.7 ± 15.35b	120.79 ± 12.71b	57.55 ± 11.95
ALB (g/dL)	2.41 ± 0.3a	2.23 ± 0.17a	2.1 ± 0.49a	1.6 ± 0.61b	2.65 ± 0.30
TP (g/dL)	6.88 ± 0.61a	6.80 ± 0.30a	5.11 ± 0.5a	3.71 ± 0.46b	5.75 ± 0.87
BILT (mg/dL)	0.09 ± 0.01a	0.11 ± 0.02a	0.10 ± 0.01a	0.30 ± 0.05b	0.02 ± 0.18c
GLUC (mg/dL)	120.9 ± 16.14a	114.14 ± 17.03a	127.57 ± 30.63a	117.80 ± 3.0a	138.72 ± 30.1b
TRIG (mg/dL)	47.4 ± 10.93a	42.82 ± 12.14a	41.2 ± 10.36a	40.82 ± 7.0a	46.87 ± 18.73a

AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALB = albumin, TP = total protein, BILT = total bilirubin, GLUC = glucose, TRIG = triglycerides; a,b,c = Means on the same line followed by equal letters are statistically equal, paired Student's t-test, # Wilcoxon test ($p<0.05$).

absence of red propolis hepatotoxicity, as well as underlines its hepatoprotective effect.

This study also showed the significant increase in serum bilirubin levels in G4, while in G1, G2, and G3 the values remained within the normal range for the species and did not differ from each other. No significant differences in mean glucose and triglyceride values were observed between the experimental groups (Table 5). The assessment of serum bilirubin levels can diagnose or monitor liver diseases such as cirrhosis, hepatitis or biliary obstruction (Thapa & Walia 2007). Bilirubin is a byproduct of hemoglobin metabolism in the liver, and when there is excess of this substance in the blood, it is a sign that it is not properly metabolized by the liver (Martelli 2012). Thus, the permanence of serum bilirubin values in G1, G2, and G3 could reinforce the absence of hepatotoxicity and the hepatoprotective effect of red propolis from Rio Grande do Norte.

Macroscopic and histological assessment

In the macroscopic examination of the livers collected at the end of the experimental period, the animals of G1 and G2 had a liver with the morphological aspect within the normal range observed for the species, without evidence of changes in shape, color, and consistency (Fig.1A,B). However, the animals from Groups G3 and G4 developed cirrhosis. The livers of the animals of G3 presented surface containing small nodules flush with the organ surface and thin capsule, and relatively soft consistency and whitish-yellow color (Fig.1C). The livers of the animals from G4 showed evidence of evolution of severe cirrhosis, characterized by the presence of large regenerative nodules on the surface, thickened capsule, hardened and cut resistant consistency, and brownish coloration (Fig.1D).

In a histopathological assessment performed on hematoxylin-eosin (HE) stained slides, the liver tissue of the animals from G1 and G2 had no changes, showing normal histological appearance (Fig.2A,B). In the liver of G3 animals, hepatocyte necrosis was observed, characterized by the presence of cells with a pycnotic nucleus, in cariorrexia and cytoplasmic acidophilia. Hepatocyte necrosis was more evident in the central lobular and periportal regions. Isolated foci of hepatocyte hyperplasia, discrete connective tissue deposition around the central lobular vein, as well as thin connective tissue septa involving hepatocyte nodules and moderate bile duct hyperplasia (Fig.2C) were also found. The histological changes were more severe in the liver of G4 animals, with the presence of large regenerative nodules of hepatocytes surrounded by thick bridges of connective tissue, and deposition of connective tissue in the periportal

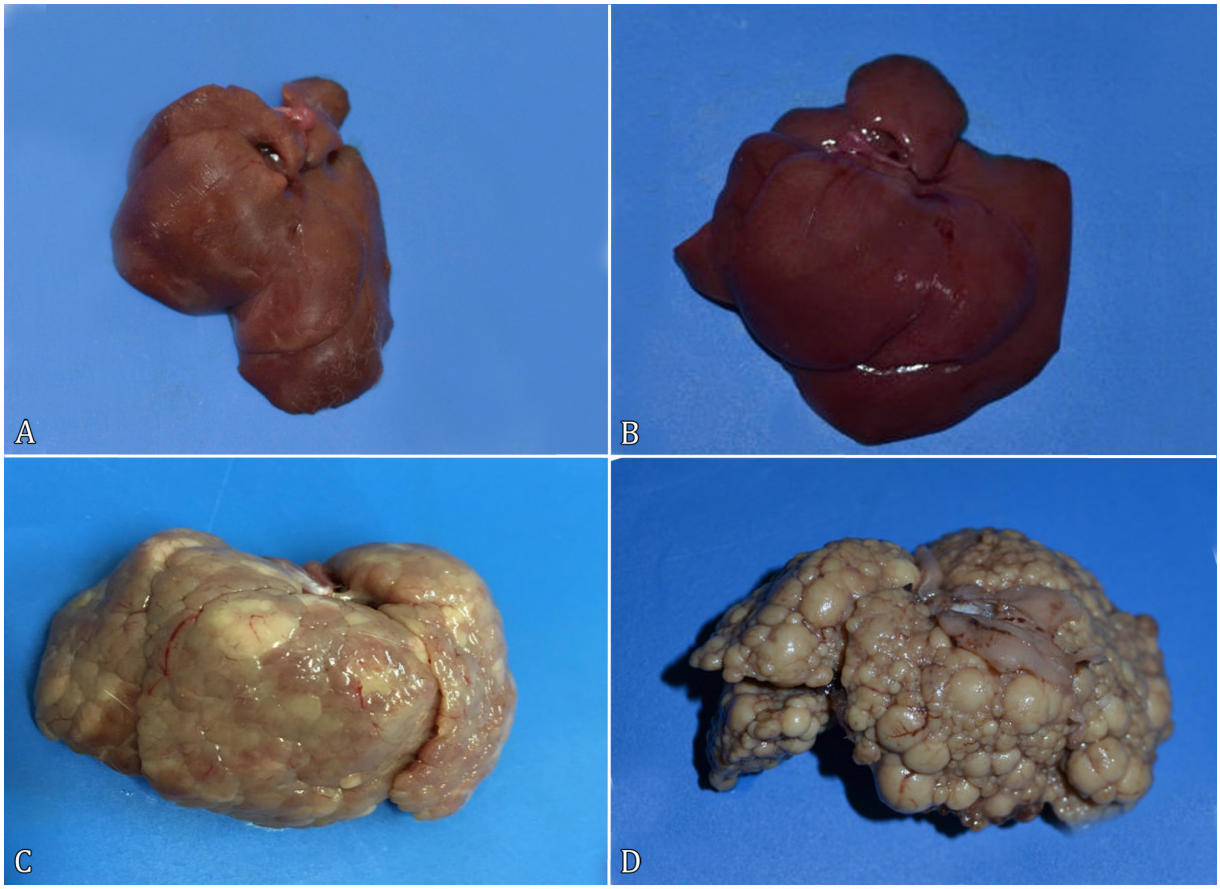


Fig.1. (**A**) Rodent liver *Rattus norvegicus*, Wistar strain, Group G1, control, with normal morphological aspects. (**B**) Rodent liver *Rattus norvegicus*, Wistar strain, group G2, treated with ethanolic extract of red propolis, with normal morphological aspects. (**C**) Cirrhotic rodent liver, *Rattus norvegicus*, Wistar strain, group G3, with small regenerative nodules flush with the surface. G3 rodents were treated with ethanolic extract of red propolis and thioacetamide. (**D**) Rodent cirrhotic liver, *Rattus norvegicus*, Wistar strain, group G4, with large regenerative nodules on the surface. G4 rodents were treated with thioacetamide.

region and around the central-lobular vein and moderate bile duct hyperplasia (Fig.2D).

Big advances in understanding the mechanisms responsible for hepatotoxic damage have emerged from *in vitro* or *in vivo* model studies. Such models also allowed proving the therapeutic and toxic potentialities of drugs or natural products. Pathological damage caused by hepatotoxins in experimental models may be similar to the damage that occurs in different animal and human liver diseases (Yogalakshmi et al. 2010). Several animal models reproduce both surgical and chemical liver damage. Chemical procedures are developed through the administration of hepatotoxic substances. Among several experimental models of cirrhosis induction, TAA, a drug originally used as a fungicide to protect oranges against deterioration and later recognized as a potent hepatotoxin, has been the most promising because it produces macroscopic and histological morphological patterns similar to cirrhosis in human beings (Passos et al. 2010).

The study of the hepatotoxic and hepatoprotective potential of a drug or natural products can be assessed by diagnosing liver lesions, by studying changes in serum biochemical profile, and can be confirmed by anatomopathological examination of the liver (Garcia et al. 2004b).

Thus, elevated levels of AST, ALT, and bilirubin in the blood together with liver histological changes such as hepatocyte degeneration, necrosis and hyperplasia, and fibrosis are indicators of AAT-induced hepatocellular damage. In the livers of animals of the group treated with TAA (G4), a loss of the organ architectural pattern was observed, with the presence of regenerative nodules characteristic of cirrhosis. Cirrhosis is the scar response of the liver in most chronic or advanced inflammatory liver diseases, which is anatomically defined

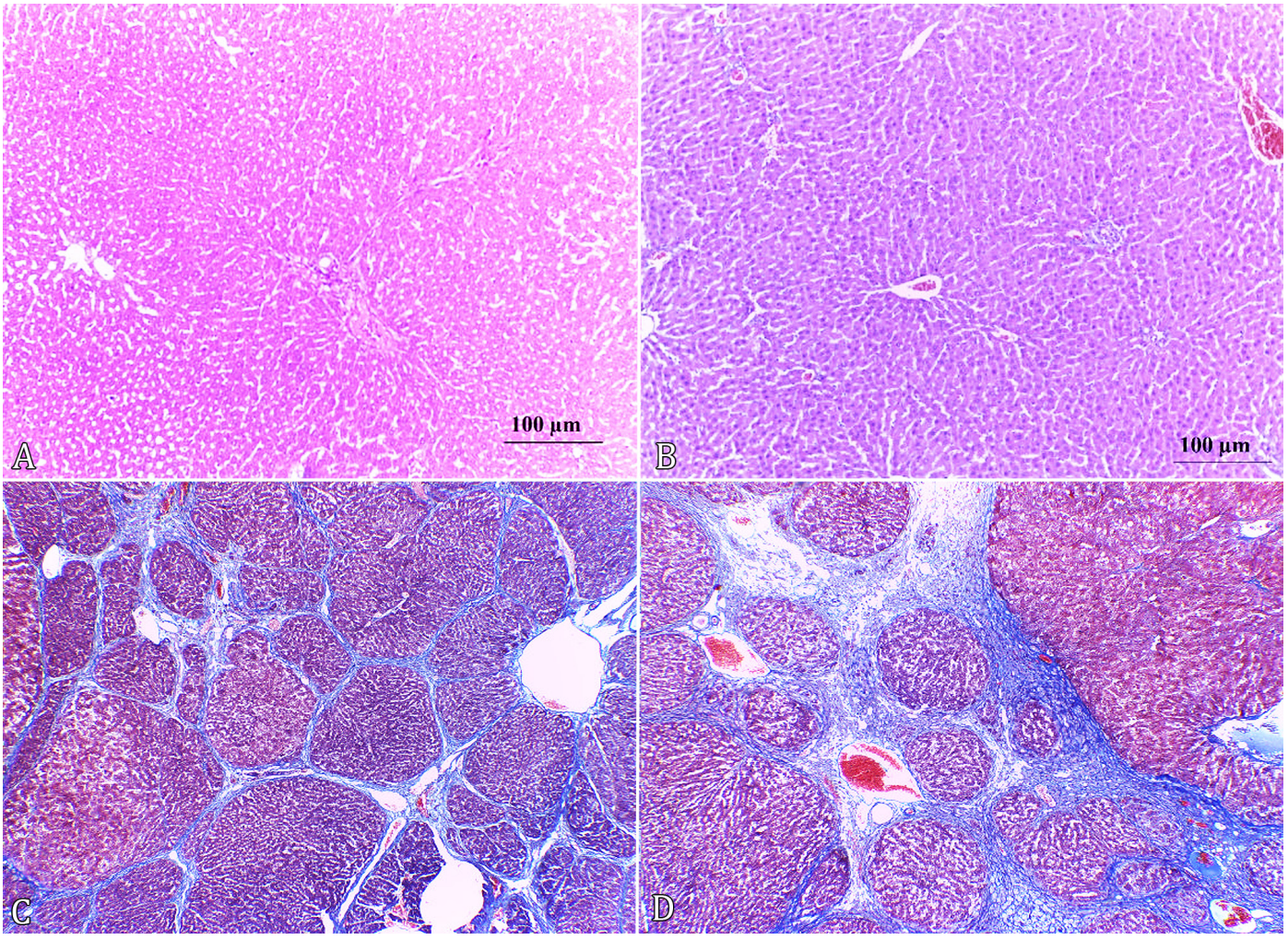


Fig.2. (**A**) Rodent liver *Rattus norvegicus*, Wistar strain, Group G1, control, with normal histological appearance. HE, obj.20x. (**B**) Rodent liver Rattus norvegicus, Wistar strain, Group G2, treated with ethanolic extract of red propolis, with normal histological appearance. Gomori's trichrome, obj.20x. (**C**) Rodent liver, *Rattus norvegicus*, Wistar strain, from Group G3, with regenerative nodules surrounded by thin connective tissue septa. G3 rodents were treated with ethanolic extracts of propolis and thioacetamide. HE, obj.20x. (**D**) Rodent liver, *Rattus norvegicus*, Wistar strain, Group G4, with regenerative nodules surrounded by thick fibrous connective tissue septa. G4 rodents were treated with thioacetamide. HE, obj.20x.

as a diffuse process of fibrosis and nodule formation, often accompanied by hepatocellular necrosis (Brandão et al. 2006).

Thus, the results of the evaluation of the hepatoprotective effect of red propolis of Rio Grande do Norte are promising, in the reduction of liver damage experimentally induced by hepatotoxic substances. The macroscopic and histological evaluation of the livers of G3 animals showed that the red propolis ethanolic extract had a hepatoprotective effect, reducing the severity of cirrhosis because the characteristic regenerative nodules of cirrhosis were smaller and more discreet when compared to G4 animals. In G3 animals, the connective tissue deposition was less intense, and the septa surrounding the regenerative nodules were thin and little evident (Fig.2C).

Liver weight

The parameter of the liver weight was statistically similar between G1 and G2. There was a significant increase in liver weight in G3 and G4 compared to the other groups (Table 6). The increase in liver weight in G3 and G4 than in the other groups is probably related to the pathological characteristic aspects of cirrhotic liver tissue. The main pathological processes that occur in cirrhosis are cell fibrosis, regeneration, or proliferation (Ivanova 2016), so liver hypertrophy often occurs as observed in the livers of animals from G3 and G4.

Stereological analysis

Liver tissue analysis in the different experimental groups by stereology showed that there was no statistical difference between the percentage of hepatocytes in G1 and G2. However, the percentage of hepatocytes in G4 decreased significantly compared to the other groups, while the percentage of hepatocytes in G3 did not differ from G1, G2. There was also a significant reduction in the percentage of sinusoids in G4, whereas, in G1, G2 and G3 did not differ significantly. Regarding the percentage of collagen in the liver tissue samples, there was no significant difference in G1 and G2. In G3 and G4, there was a significant increase in the percentage of collagen and a significant difference than the other groups (Table 7).

This study sought to analyze the histological images of liver tissue by the stereological method. This method can determine three-dimensional quantitative parameters of anatomical structures from two-dimensional histological sections. Stereology is a tool that provides excellent results as it provides reliable data for quantitative analysis of the material of histological origin (Ochs 2006). This method helps to verify the histological changes of the livers of rodents in the different experimental groups. Therefore, the reduction in the percentage of G4 hepatocytes may be attributed to the detrimental effect of TAA on these cells, while the increase in the percentage of hepatocytes in G3 relative to G4 may indicate that the red propolis ethanolic extract protected the hepatocytes from the TAA deleterious effect or possibly the ethanolic extract of red propolis may have contributed to the regeneration of damaged hepatocytes.

Hepatocytes are the main parenchymal cells of the liver and are capable of performing almost all functions assigned to this organ, considered as the most multifunctional cell in the body (Harzer et al. 2015). Thus, the absence of statistical difference in the percentage of hepatocytes in G3 than in G1 and G2 and significant increase than in G4 may indicate a morphological improvement that may result in a functional liver improvement, confirming once again the hepatoprotective effect of the ethanolic extract of red propolis.

Changes in the percentage of sinusoids also reflect the histological changes in the liver tissue of rodents submitted to different experimental protocols. Hepatic sinusoids are capillaries or channels located in the space between the hepatocyte cords, in which blood passes to reach the terminal hepatic vein. Its walls are lined with fenestrated endothelial cells that expose hepatocytes directly to a series of hormones, growth factors, and nutrients that have hepatotropic action (Jesus et al. 2000). The significant reduction in the percentage of sinusoids in G4 can be explained by the establishment of the most intense fibrosis in this group. According to Bedossa et al. (2003), the death of hepatocytes leads to fibrosis repair, with deposition of fibrous tissue, which will occur around the sinusoid vessels, causing obliterative fibrosis, which may result in compromised intrahepatic blood and biliary flow. This set of characteristics reduces blood contact with hepatocytes,

Table 6. Mean ± standard deviation values of rodent's liver weight (*Rattus norvegicus* Berkenhout, 1769), Wistar strain in experimental groups G1 (control), G2 (treated with ethanolic extract of red propolis), G3 (treated with ethanolic extract of red propolis and TAA) and G4 (TAA treated)

Variable	Experimental groups			
	G1	G2	G3	G4
Weight (g)	10.05 ± 2.74a	8.73 ± 0.64a	15.98 ± 1.9b	17.01 ± 0.89b

a,b = Means on the same line followed by equal letters are statistically equal, paired Student's t-test, # Wilcoxon test ($p<0.05$).

Table 7. Mean ± standard deviation values of stereological parameters of rodent liver (*Rattus norvegicus* Berkenhout, 1769), Wistar strain, in experimental groups G1 (control), G2 (treated with ethanolic extract of red propolis), G3 (treated with ethanolic extract of red propolis and TAA) and G4 (treated with TAA)

Variables (%)	Experimental groups			
	G1	G2	G3	G4
Hepatocytes	77.64 ± 5.91a	77.47 ± 3.18a	64.60 ±0.50ab	55.68 ± 3.70b
Sinusoids	19.01 ± 5.01a	18.02 ± 5.31a	17.29 ± 8.48a	9.78 ± 4.78b
Collagen	3.35 ± 1.49a	4.45 ± 4.78a	18.11 ± 7.98b	34.54 ± 5.22c

a,b,c = Means on the same line followed by equal letters are statistically equal, paired Student's t-test, # Wilcoxon test ($p<0.05$).

which can affect the availability of hepatocyte nutrients and hepatotrophic factors and impair their function.

Changes in collagen percentages may also be related to the pathological features of cirrhotic liver tissue since the literature conceptualizes cirrhosis as the final phase of chronic liver damage in response to direct aggression or inflammation, with a large number of fibrous tissue forms, specifically collagen fibers deposited, thus the significant increase in collagen in G4 clearly reflects this condition. The significant reduction in the percentage of G3 collagen than in G4 also reflects the hepatoprotective effect of ethanolic extract of red propolis from Rio Grande do Norte, reducing hepatocyte damage, reparative fibrosis, and maintenance of the histological pattern close to observed in normal liver tissue, as found in Groups G1 and G2.

CONCLUSIONS

The hepatoprotective effect of red propolis was found in Wistar rodents (*Rattus norvegicus*), treated with 500mg/kg of ethanolic propolis extracts and experimentally submitted to induction of cirrhosis by TAA, since the results of serum levels of the enzymes indicating liver damage showed that the ethanolic extract did not cause liver toxicity and exerted hepatoprotective effect.

Changes indicating cirrhosis were less significant in macroscopic and histological evaluations in animals treated with propolis extract. *In vitro*, the extract promoted genoprotective effect by reducing DNA damage in liver cells damaged by H_2O_2, as well as exerting no cytotoxic effect on normal cells (L929), showing antineoplastic activity in human hepatocellular carcinoma cell line (HepG2), in which the red propolis is a promising candidate to inhibit cell growth and contribute to the different steps related to the carcinogenesis process.

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

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Antimicrobial susceptibility and minimal inhibitory concentration of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca[1]

Carolina S.G. Pereira[2], Luís Felipe C. Zulim[2], Rogério Giuffrida[2], Aline G. Cruz[3], Bruna T.D. Foglia[3], Hugo Benguella[3], Aline S. Batista[4] and Silvia F. Andrade[5]*

ABSTRACT.- Pereira C.S.G., Zulim L.F.C., Giuffrida R., Cruz A.G., Foglia B.T.D., Benguella H., Batista A.S. & Andrade S.F. 2019. **Antimicrobial susceptibility and minimal inhibitory concentration of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca.** *Pesquisa Veterinária Brasileira 39(9):757-763*. Departamento de Clínica Médica de Pequenos Animais, Hospital Veterinário, Universidade do Oeste Paulista, Rod. Raposos Tavares Km 572, Presidente Prudente, SP 19067-175, Brazil. E-mail: silviafranco@unoeste.br

The objective of this study was to evaluate the antimicrobial susceptibility profile of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca (KCS). We evaluated 65 dogs diagnosed with KCS and 30 healthy dogs (Control Group). Conjunctival swab samples were collected after KCS was diagnosed. Microbiological examinations were performed, including aerobic culture, antimicrobial susceptibility testing and minimum inhibitory concentration (MIC) determination for chloramphenicol, tobramycin, ofloxacin and moxifloxacin. MICs of the fifteen most resistant strains of *Staphylococcus pseudintermedius* (*Staphylococcus intermedius* Group, SIG) and the fifteen most resistant strains of gram-negative bacteria were determined. By percentage, the microorganisms exhibited the highest susceptibility to polymyxin B, tobramycin and chloramphenicol and the lowest to tetracycline. Three multi-drug-resistant strains of SIG were detected: one displayed isolated susceptibility to cefazolin, another to vancomycin, and another to polymyxin B and amikacin. The species of bacteria isolated from the eyes of dogs with KCS presented variable susceptibility to the antibiotics tested. We found evidence of the emergence of quinolone-resistant strains of SIG and further evidence of increased ocular prevalence. These findings reinforce the need to identify the bacteria involved and their antimicrobial susceptibility profile, as secondary infections can serve as exacerbating and perpetuating factors in KCS.

INDEX TERMS: Antimicrobial susceptibility, testing, disk diffusion, bacteria, dogs, eyes, keratoconjunctivitis sicca, bacterioses.

RESUMO.- [Susceptibilidade antimicrobiana e concentração inibitória mínima de bactérias isoladas dos olhos de cães com ceratoconjuntivite seca.] O objetivo deste estudo foi avaliar o perfil de susceptibilidade antimicrobiana de bactérias isoladas de olhos de cães com ceratoconjuntivite seca (CCS). Foram avaliados 65 cães com diagnóstico de CCS e 30 cães saudáveis (Grupo Controle). Depois do diagnosticado de CCS, suabes conjuntivais foram coletados. Exames microbiológicos foram realizados, incluindo cultura aeróbia, teste de susceptibilidade antimicrobiana e determinação da concentração inibitória mínima (CIM) para cloranfenicol, tobramicina, ofloxacina e moxifloxacina. Para determinar a CIM, foram selecionadas as quinze cepas mais resistentes de *Staphylococcus pseudintermedius* (*Staphylococcus intermedius* Group-SIG) e as quinze cepas mais resistentes de bactérias gram-negativas. Os microrganismos apresentaram maior suscetibilidade percentual à polimixina B, tobramicina e cloranfenicol e menor suscetibilidade à tetraciclina.

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[2] Graduate Studies Program in Animal Science, Universidade do Oeste Paulista (Unoeste), Rod. Raposo Tavares Km 572, Presidente Prudente, SP 19067-175, Brazil.

[3] Faculdade de Medicina Veterinária, Unoeste, Rod. Raposo Tavares Km 572, Presidente Prudente, SP 19067-175.

[4] Residente do Laboratório de Patologia Clínica, Hospital Veterinário, Unoeste, Rod. Raposo Tavares Km 572, Presidente Prudente, SP 19067-175.

[5] Departamento de Clínica Médica de Pequenos Animais, Hospital Veterinário, Unoeste, Rod. Raposos Tavares Km 572, Presidente Prudente, SP 19067-175.
*Correspondig author: silviafranco@unoeste.br

Três cepas de SIG resistentes a múltiplos medicamentos foram detectadas, do quais um demonstrou suscetibilidade isolada à cefazolina, outro à vancomicina e outro à polimixina B e à amicacina. As espécies de bactérias isoladas dos olhos de cães com CCS apresentaram suscetibilidade variável aos antibióticos testados. Encontramos evidências do surgimento de cepas resistentes à quinolona de *S. pseudintermedius* e outras evidências de aumento da prevalência ocular. Esses achados reforçam a necessidade de identificar as bactérias envolvidas e seu perfil de susceptibilidade aos antimicrobianos, pois as infecções secundárias podem servir como fatores exacerbantes e perpetuantes na CCS.

TERMOS DE INDEXAÇÃO: Susceptibilidade antimicrobiana, concentração inibitória mínima, bactérias, olhos, cães, ceratoconjuntivite seca, caninos, bacterioses.

INTRODUCTION

Keratoconjunctivitis sicca (KCS), or dry eye, is a common ophthalmopathy that usually occurs in dogs and humans due to a deficiency in the aqueous component of the pre-corneal tear film or to modified quality of and/or reduction in tear stability. KCS has multiple etiologies, and the most prevalent involves immunomediation (Carter & Colitz 2002, Williams 2008, McGinnigle et al. 2012, Stevenson et al. 2012, Giuliano 2013, Messmer 2015).

Topical therapy for keratoconjunctivitis sicca generally consists of immunosuppressants, such as cyclosporine, tacrolimus or pimecrolimus, and lachrymal substitutes, which are combined with antibiotics for the treatment of secondary infections. Typical antibiotics include gentamicin, tobramycin, chloramphenicol, ofloxacin, moxifloxacin, and gatifloxacin. In addition, topical anti-inflammatory agents, mucolytics and parasympathomimetic stimulants of lacrimal secretion may be used (Wilkie 1996, Carter & Colitz 2002, Miller 2008, Giuliano 2013, Messmer 2015).

Secondary bacterial infections are common in keratoconjunctivitis sicca (Carter & Colitz 2002, Messmer 2015, Leigue et al. 2016). In general the ocular surface is colonized by commensal bacteria that, together with humoral factors, inhibit colonization by pathogenic microorganisms. In healthy dogs, gram-positive bacteria are principally present on the ocular surface. Nonetheless, species profiles vary according to geographical location, technique used to cultivate tear secretions, contact with other animals, population density and season (Armstrong 2000, Prado et al. 2005, Gould & Papasouliotis 2013).

In eyes with an external injury, superficial inflammation of the cornea and conjunctiva is triggered, leading to a decrease in antibacterial enzymes (lactoferrin, lysozyme and peroxidase). Moreover, the accompanying loss of epithelial integrity, depending on the degree and intensity of the trauma and tear film modifications, favors the colonization of ocular structures by opportunistic bacteria and fungi (Murphy et al. 1978, Mannis & Smolin 1996, Armstrong 2000).

Microorganisms with a high prevalence in the eyes of healthy dogs include *Staphylococcus epidermidis*, *Staphylococcus pseudintermedius*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Escherichia coli*, *Streptococcus* sp., *Enterobacter* sp., and *Pseudomonas aeruginosa* (Murphy et al. 1978, Andrade et al. 2002, Prado et al. 2005). In the ocular environment, these microorganisms usually act as opportunists, aggravating the clinical signs of keratoconjunctivitis sicca. Under these circumstances, empiric treatment with eye drops containing high concentrations of antimicrobials such as chloramphenicol, aminoglycosides (neomycin, gentamicin, tobramycin), tetracyclines, second-generation (ofloxacin, ciprofloxacin) and fourth-generation (gatifloxacin and moxifloxacin) quinolones are indicated (Lin & Petersen-Jones 2007, Quinn et al. 2011, Gould & Papasouliotis 2013, Mello et al. 2017). However, the efficacy of topical therapy may be influenced by several factors, including antimicrobial resistance of the microorganisms involved, insufficient penetration of the antimicrobial into the inner areas of the eye (vitreous humor), co-infection with fungal microorganisms, changes in microorganism profiles during treatment and immune-debilitating conditions (Hendrix & Cox 2008, Kaye et al. 2010, Ahn et al. 2011).

Some studies have suggested that *in vitro* susceptibility may predict clinical outcome in humans with bacterial keratitis (Kaye et al. 2010, Lalitha et al. 2012). To date, determination of the minimum inhibitory concentration (MIC), which corresponds to the lowest antimicrobial concentration capable of inhibiting the development of the bacterium *in vitro,* is recognized as the most reliable method for selecting antimicrobial drugs to treat eye infections (Andrews 2001, CLSI 2017). Indeed, a recent study reported the MICs of *Pseudomonas aeruginosa* isolated from septic ocular surface disease in different animal species, and MIC results were used to target antimicrobial therapy strategies, focusing on the concentration and frequency of tobramycin application (Leigue et al. 2016).

The aim of the present study was to evaluate the antimicrobial susceptibility profile of bacteria isolated from the eyes of dogs with keratoconjunctivitis sicca in the region of Presidente Prudente/SP, Brazil.

MATERIALS AND METHODS

Ethics statement. The study was conducted and approved in accordance with the animal testing regulations of the Ethics Committee on Animal Use (CEUA) of Unoeste (Protocol no. 1802 and 1803) and was in accordance with the rules of the ARVO (Association for Research in Vision and Ophthalmology - Statement for the use of animals in ophthalmic and visual research).

Animals. This study was conducted at the Veterinary Hospital of the Unoeste, Presidente Prudente/SP, from 2014 to 2017. We evaluated 65 dogs (130 eyes) diagnosed with keratoconjunctivitis sicca; 36 (55%) were female, and 29 (45%) were male, with a mean age of 7.1±3.9 years old and a mean weight of 10.4±7.5kg. The most common breeds were Lhasa Apso and mixed breeds, with 17 (26.2%) dogs each, followed by 10 Poodles (15.4%), 5 Pinschers (7.7%), 3 Yorkshire Terriers (4.6%), 2 Pit Bulls (3.1%) and 11 other breeds (1.5%). The dogs were registered through an authorization form (Term of Free and Informed Consent) signed by the owners and those responsible for the project. The control group consisted of 30 healthy dogs (60 eyes) without ocular problems from the kennel of Unoeste institution; 17 (56.6%) were female, and 13 (43.4%) were male, with a mean age of 4.3±3.0 years old and a mean weight of 18.1±12.7kg. The breeds of the control group were 24 (80%) mixed breed, 5 (16.7%) Boxer and 1 (3.3%) Labrador Retriever.

Ophthalmic exams. All animals were evaluated using ophthalmic exams: biomicroscopy slit-lamp examination (Kowa SL-17 portable slit lamp, Japan), Schirmer tear test 1 (STT-1) (Teste de Schirmer, Ophthalmos, São Paulo, Brazil), fluorescein test (FT) (Flouresceína

Strips, Ophthalmos, São Paulo, Brazil), tear film break-up time (TBUT), and applanation tonometry (Tono-Pen, USA). The diagnosis of KCS was based on clinical signs (conjunctival hyperemia, ocular discharge and presence of corneal opacity, ulcer, vascularization or pigmentation) and specific ophthalmic tests that included STT-1 (values ≤15mm/min) and/or TBUT (≤25 seconds) (Maggs 2008). Only dogs with keratoconjunctivitis sicca and no history of having received systemic or topical antibiotics in the preceding 30 days were included in the study.

Microbiological methods. After keratoconjunctivitis sicca was diagnosed, samples of ocular secretions were collected from the lower conjunctivae of both eyes using sterile cotton swabs, which were used to inoculate Petri dishes containing 5% defibrinated bovine blood agar and MacConkey agar. The inoculated plates were incubated under aerobic conditions at 37°C for 24-48 hours. Isolated bacterial colonies were classified according to morphology, staining and biochemical characteristics. Only strains isolated from dogs with KCS were tested for antimicrobial susceptibility. Initially, the isolates for susceptibility testing were seeded in brain-heart infusion broth and incubated at 37°C for 24-48 hours under aerobic conditions. After this period, the broths were diluted with saline until reaching a turbidity of 0.5 on the McFarland scale. A sterile cotton-wool swab was dipped into the suspension, and excess liquid was removed by rotating the swab against the side of the container. The inoculum was then spread evenly over the entire surface of a plate by swabbing in five directions (Quinn et al. 2011).

After the 30-minute period necessary for inoculum absorption, filter-paper disks (Cecon, São Paulo, Brazil) were deposited evenly over the agar surface. These disks contained the following antibacterial agents: cefazolin (30mcg), ciprofloxacin (5mcg), chloramphenicol (30mcg), gentamicin (10mcg), neomycin (30mcg), norfloxacin (10mcg), ofloxacin (5mcg), polymyxin B (300mcg), tetracycline (30mcg), tobramycin (10mcg) and vancomycin (30mcg). After 18 hours of incubation, the diameters of the inhibition zones around the disks were measured with a caliper, and the results, expressed in millimeters, were compared to international standards for the interpretation of results (CLSI 2017). For drugs without criteria for dogs, standards of interpretation indicated for humans were used.

The MICs of the most relevant and potentially pathogenic isolates for dogs were evaluated for sensitivity to chloramphenicol, tobramycin, ofloxacin and moxifloxacin through the cutoffs by the E-test method (Probac, São Paulo, Brazil). One aliquot of the broth was seeded superficially onto Mueller-Hinton agar plates using a sterile swab. After 30 minutes, commercial plastic strips impregnated with chloramphenicol (0.016-256µg/mL), tobramycin (0.064-1024µg/mL), or ofloxacin and moxifloxacin (0.002-32µg/mL) were deposited onto the agar surface. After incubation at 37°C for 24 hours, the observed points of growth inhibition corresponding to the MICs were computed for each agent. The patterns observed were compared with the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI 2017) to classify isolates as susceptible, partially susceptible or resistant. Given that CLSI (2017) has no cutoff for moxifloxacin, we adopted the breakpoints recommended by Eucast. Cutoff points for susceptibility were as follows: chloramphenicol, S ≤8µg/mL; moxifloxacin, S ≤0.25µg/mL; ofloxacin, S ≤1µg/mL; and tobramycin S ≤4µg/mL. The cutoff points for gram-negative bacteria (Enterobacteriaceae) were as follows: chloramphenicol, S ≤8µg/mL; moxifloxacin, S ≤0.5µg/mL; ofloxacin, S ≤1µg/mL; and tobramycin, S ≤4µg/mL.

Statistical analysis. The prevalence of positive ocular culture was determined for both the dogs with keratoconjunctivitis sicca and those in the control group using 95% confidence intervals (CIs). The positivity percentages of dogs in the KCS and control groups were compared using the binomial test for proportions. The significance level used in all tests was 5%. The categorical concordance index was calculated to evaluate agreement between the results that classified the isolates as sensitive and resistant.

RESULTS

In the group of animals with keratoconjunctivitis sicca, 59 of the 65 dogs (90.8%, 95% CI = 83.7-97.8%) were positive by culture for at least one eye. In the control group, 11 of the 30 dogs (36.7%, 95% CI = 19.4-53.9%) were culture positive for at least one eye. These percentages were significantly different between the control and KCS groups (p<0.0001).

All isolates from the control group were classified as *Staphylococcus pseudintermedius*. However, because this species presents phenotypic and genotypic similarities with species of the complex 'Staphylococcus intermedius Group (SIG)', we chose to call it "SIG" (Mališová et al. 2019). For animals with keratoconjunctivitis sicca, the microbiological profile consisted of both gram-positive and gram-negative species (Table 1). A total of 7.5% of the microorganisms were isolated from only one eye, whereas 92.4% were found in both eyes. Discordance between the microbiological results for the right and left eyes was observed in 44 (67.7%) of the 65 dogs with KCS.

The susceptibility based on the disk-diffusion method of the agents isolated is described in Table 2. To determine MICs through the E-test method, fifteen SIG strains and fifteen gram-negative strains (eleven *Escherichia coli*, three *Enterobacter aerogenes* and one *Citrobacter freundii*) were selected (Table 3). High agreement was verified between the disk-diffusion and E-test methods for chloramphenicol, good agreement for ofloxacin and weak agreement for tobramycin (Table 4).

SIG strains exhibiting multi-drug resistance were detected in 3 (4.6%) of the 65 dogs with keratoconjunctivitis sicca; this was defined as resistance to nine or more principal antimicrobials. These isolates were subjected to new susceptibility tests, which revealed that one isolate was susceptible to cefazolin, one was susceptible to vancomycin, and one was susceptible to polymyxin B and amikacin. Due to the absence of suitable commercial preparations in Brazil,

Table 1. Microorganisms isolated from ocular secretions from both eyes of dogs (n = 65) with KCS

Microorganism	Total (%)
Burkholderia cepacia	1 (0.8)
Citrobacter freundii	1 (0.8)
Corynebacterium sp.	1 (0.8)
Enterobacter gergoviae	1 (0.8)
Klebsiella pneumoniae	1 (0.8)
Serratia sp.	1 (0.8)
Proteus mirabilis	2 (1.5)
Pseudomonas aeruginosa	3 (2.3)
Enterobacter aerogenes	4 (3.0)
Escherichia coli	14 (10.6)
Streptococcus sp.	15 (11.4)
Staphylococcus pseudintermedius	88 (66.7)
TOTAL	132 (100)

Table 2. Proportion susceptible to antimicrobial drug using the disk diffusion method

Microorganism	Susceptibility									
	Cfz	Cip	Clo	Gen	Neo	Nor	Ofx	Pol B	Tet	Tob
	n = 60	n = 132	n = 132	n = 132	n = 132	n = 132	n = 132	n = 72	n = 132	n = 132
Burkholderia cepacia	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	Nt	1/1 (100%)	1/1 (100%)
Corynebacterium sp.	1/1 (100%)	0/1 (0%)	1/1 (100%)	1/1 (100%)	1/1 (100%)	0/1 (0%)	0/1 (0%)	Nt	0/1 (0%)	1/1 (100%)
Pseudomonas aeruginosa	3/3 (100%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	0/3 (0%)	3/3 (100%)	3/3 (100%)	Nt	0/3 (0%)	3/3 (100%)
Enterobacteriaceae	10/12 (83.3%)	14/24 (58.3%)	19/24 (79.2%)	15/24 (62.5%)	11/24 (45.8%)	17/24 (70.8%)	20/24 (83.3%)	13/13 (100%)	11/24 (45.8%)	16/24 (66.7%)
Staphylococcus spp.	20/31 (64.5%)	55/88 (62.5%)	80/88 (90.9%)	70/88 (79.5%)	56/88 (63.6%)	61/88 (69.3%)	58/88 (65.9)	57/58 (98.3%)	34/88 (38.6%)	76/88 (86.4%)
Streptococcus spp.	8/12 (66.7%)	8/15 (53.3%)	7/15 (46.7%)	14/15 (93.3%)	5/15 (33.3%)	13/15 (86.7%)	14/15 (93.3%)	1/1 (100%)	7/15 (46.7%)	10/15 (66.7%)
TOTAL	43/60 (71.6%)	81/132 (61.3%)	108/132 (81.8%)	104/132 (78.8%)	74/132 (56.1%)	97/132 (73.5%)	96/132 (72.7%)	71/72 (98.6%)	53/132 (40.2%)	109/132 (82.6%)

Cfz = cefazolin, Cip = ciprofloxacin, Clo = chloramphenicol, Gen = gentamicin, Neo = neomycin, Nor = norfloxacin, Nt = not tested, Ofx = ofloxacin, Pol B = polymyxin B, Tet = tetracycline, Tob = tobramycin.

Table 3. Sensitivity of microorganisms to the antibiotics using the E-test method

Concentration µg/mL	Antimicrobial							
	Staphylococcus pseudintermedius (n=15)				Gram-negative (Enterobacteriaceae) (n=15)			
	Clo	Mox	Ofx	Tob	Clo	Mox	Ofx	Tob
0.016	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	0/15 (0%)
0.047	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	0/15 (0%)
≤0.064	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	4/15 (26.7%)	1/15 (6.7%)	0/15 (0%)
0.094	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	1/15 (6.7%)	2/15 (13.3%)	0/15 (0%)
0.125	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	4/15 (26.7%)	3/15 (20%)	0/15 (0%)
0.19	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	2/15 (13.3%)	4/15 (13.3%)	0/15 (0%)
0.25	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7.6%)	2/15 (13.3%)	1/15 (6.7%)
0.38	0/15 (0%)	0/15 (0%)	2/15 (13.3%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	4/15 (26.7%)
0.50	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	6/15 (40%)
0.75	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	3/15 (20%)
1.0	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	1/15 (3.3%)	0/15 (0%)	1/15 (3.3%)	0/15 (0%)
1.5	2/15 (13.3%)	2/15 (13.3%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
2	3/15 (20%)	3/15 (20%)	1/15 (6.7%)	2/15 (13.3%)	3/15 (20%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
3	4/15 (26.7%)	4/15 (26.7%)	0/15 (0%)	1/15 (6.7%)	3/15 (20%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
4	1/15 (6.7%)	1/15 (6.7%)	0/15 (0%)	1/15 (6.7%)	2/15 (13.3%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
6	0/15 (0%)	0/15 (0%)	0/15 (0%)	4/15 (26.7%)	3/15 (20%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
8	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)
12	0/15 (0%)	0/15 (0%)	0/15 (0%)	1/15 (6.7%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
24	3/15 (20%)	3/15 (10%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
≥32	2/15 (13.3%)	2/15 (13.3%)	11/15 (73.3%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
≥256	0/15 (0%)	0/15 (0%)	0/15 (0%)	0/15 (0%)	3/15 (20%)	0/15 (0%)	0/15 (0%)	0/15 (0%)
Total of sensitive strains*	10/15 (66,7%)	0/15 (0%)	3/15 (20%)	9/15 (60%)	12/15 (80%)	15/15 (100%)	15/15 (100%)	14/15 (93,3%)

Clo = chloramphenicol, Mox = moxifloxacin, Ofx = ofloxacin, Tob = tobramycin; * criteria established based on serum concentrations.

Table 4. Percentage agreement of results from E-test and disk-diffusion (DD) tests, and estimates of the Kappa coefficients of agreement

Comparison	% agreement	Kappa	p	Interpretation
Tob (E-test x DD)	70.0%	0.3284	0.0263	Weak
Clo (E-test x DD)	93.4%	0.8421	< 0.0001	Excellent
Ofx (E-test x DD)	86.7%	0.7368	< 0.0001	Good

Tob = tobramycin, Clo = chloramphenicol, Ofx = ofloxacin.

manipulated compounded ophthalmic solutions containing only these antimicrobials were administered (5% cefazolin, 5% vancomycin and 10,000U/mL polymyxin B eye drops, Laboratory Ophthalmos, São Paulo, Brazil). In 100% of the treated animals, signs of ocular infection were resolved after 15 days of treatment (1 drop in both eyes four times daily).

DISCUSSION

The high percentage of dogs with keratoconjunctivitis sicca with positive cultures (90.8%) compared with that in the control group (36.7%) reinforces the importance of identifying the profile and susceptibility of bacterial agents to guide antibiotic treatment (Prado et al. 2006, Ledbetter et al. 2009, Oria et al. 2013, LoPinto et al. 2015). Although the control and KCS groups had different origins (kennel x tutor), we assume that the substantially high difference in the proportions of positivity between these groups may be more related to changes in the ocular microenvironment in dogs with KCS than in the environment of the animals. Considering that the antimicrobial susceptibility profile of gram-positive bacteria differs from that of gram-negative bacteria, definitive identification of the microbial agent by bacteriological culture or presumptive identification by ocular secretion cytology is crucial to guide antimicrobial therapy. In addition, more than 50% of the results of the microbiological cultures were discordant between the left and right eyes in the animals in our study, reinforcing the need for bilateral culture to identify the agents associated with infections. Overall, our results support the recommendation of avoiding empirical therapy without auxiliary laboratory tests (Murray et al. 2015, Gould & Papasouliotis 2013).

In our study, the pathogen with the highest prevalence in the eyes of the animals was SIG, which was isolated from approximately 67% of the samples investigated. For SIG, *Staphylococcus pseudintermedius* is the principal species of coagulase-positive, gram-positive commensal coccus found in the skin and mucous membranes of dogs and commonly colonizes the eyes of healthy dogs (Prado et al. 2006, Lin & Petersen-Jones 2007, Oria et al. 2013, LoPinto et al. 2015) as well as the eyes of dogs with corneal ulceration (Wang et al. 2008). In contrast gram-negative bacteria were isolated from at least one eye in 40% (26/65) of the dogs with keratoconjunctivitis sicca in our study. These results suggest that both gram- and gram-negative bacterial agents play an important role as opportunist pathogens in the conjunctival mucosa of dogs with KCS. In dog eyes, gram-positive cocci and non-fermenting glucose rods typically colonize mucosal and cutaneous sites, whereas gram-negative enteric rods likely originate from environmental contamination, constituting a transient microbiota in the eyes (Armstrong 2000, Wang et al. 2008).

The prevalence of SIG strains in the animals of this study is consistent with other studies in Brazil on the profile of microorganisms associated with canine corneal ulcer infections (Prado et al. 2006, Monteiro et al. 2018), which suggests that this group is equally important in secondary ocular infections in dogs with KCS. The second most common group of microorganisms was *Streptococcus* sp. (non-hemolytic), which likely has an uncertain significance in the genesis and perpetuation of ocular infections secondary to keratoconjunctivitis sicca because *Streptococcus* is less virulent than is beta-hemolytic *Streptococcus*, which is recognized as more relevant (Hindley et al. 2016). Second-generation fluoroquinolones, especially ciprofloxacin and ofloxacin, are commonly indicated for the treatment of ophthalmic infections in dogs, presumably because of their broad spectrum and good action against severe eye infections (Maggs 2008). Regardless, compared to tobramycin and chloramphenicol, we found low sensitivity to these drugs among the SIG strains in this study. This finding suggests that aminoglycosides and chloramphenicol may be more suitable for empirical or prophylactic treatment of ophthalmic infections in dogs compared with quinolones, such as ciprofloxacin, moxifloxacin and gatifloxacin, with a consistently reduced therapeutic efficacy due to increasing resistance rates observed in the last decade (Chawla et al. 2010, Hsu et al. 2013). In view of this, it is suggested that the use of fluoroquinolones, particularly second-generation agents, alone as monotherapy should be discouraged as treatment for eye infections in dogs with KCS, unless the results of eye-tracking smear cytology exclude gram-positive cocci, similar to the recommendations of other studies (Hindley et al. 2016). The isolates in the present study were highly susceptible to polymyxin B and chloramphenicol, which is consistent with the findings reported by other authors. Chloramphenicol is a broad-spectrum antibiotic that has been widely used since the emergence of methicillin-resistant staphylococci. However, the use of this drug in humans has been limited due to its toxic effects (Short et al. 2014, Mouney et al. 2015, Ruzauskas et al. 2015).

SIG strains resistant to nine or more antimicrobials were isolated from three dogs. Two isolates were resistant to oxacillin, an antimicrobial considered to be important as a marker of multi-drug resistance in human ocular infections (Hsu et al. 2013). Other authors have also reported increased resistance of SIG strains in canine ocular infections (Tajima et al. 2013, Kang et al. 2014a, 2014b). However, the isolates exhibited low susceptibility to tetracycline, with only 40.2% of the isolates being susceptible to this antibiotic. This result is consistent with previous findings reporting resistance to tetracycline in greater than 50% of SIG strains isolated from individuals with ophthalmic diseases (Chandler et al. 2010).

Susceptibility testing of the isolates using interpretive criteria of the E-test method revealed that tobramycin exhibits good effectiveness against both gram-negative and gram-positive bacteria, such as SIG strains. This finding is consistent with the results of others (Kang et al. 2014a). According to the E-test, the quinolones tested demonstrated low effectiveness against SIG strains, with resistance rates greater than 80%. Considering that moxifloxacin and ofloxacin have recently frequently been used clinically for the treatment of canine ophthalmopathies, our results point to the possible emergence of resistant strains among animals in the region of Presidente Prudente/SP, Brazil, in a manner similar to other regions of the world (Kang et al. 2014b, Moodley et al. 2014).

In contrast, gram-negative bacteria were found to be susceptible to the quinolones tested, suggesting that the selection pressure on these microorganisms is reduced compared with that observed for SIG strains. However, multi-drug-resistant *E. coli* strains commonly observed in urinary infections are exceptions to this finding (Cummings et al. 2015).

Given that our criteria for interpreting the results of antimicrobial tests were based on the guidelines used

for systemic antibiotics (CLSI 2017) and that the topical antimicrobial concentration generally exceeds the systemic concentration, it is possible that the results regarding susceptibility and resistance may be associated with a tendency toward underestimation of the real therapeutic efficacy of these antimicrobials. Nonetheless, some human studies have recommended the use of interpretation criteria for systemic doses of antimicrobials to guide topical therapy due to the relationship between *in vitro* sensitivity and clinical recovery of treated patients (Parmar et al. 2006, Chen et al. 2008). Thus, criteria based on systemic doses of antimicrobials are the least reasonable option when determining topical therapy in ocular infections.

CONCLUSIONS

Based on our results, bacterial species isolated from the eyes of dogs with keratoconjunctivitis sicca exhibit variable susceptibility to the various antibiotics tested.

The high frequency of quinolone-resistant strains of SIG isolates found at a high prevalence in the eyes of these animals reinforces the need to identify the antimicrobial susceptibility profile of the bacteria involved, as secondary infections can be an exacerbating and perpetuating factor in KCS.

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Glucose and insulin curve in pregnant mares and its relationship with clinical and biometric features of newborn foals[1]

Mariana A. Mousquer[2]*, Amanda B. Pereira[2], Ilusca S. Finger[3], Helen C. Franz[2], Aníbal J. Torres[4], Vitória Müller[2] and Carlos E.W. Nogueira[2]

ABSTRACT.- Mousquer M.A., Pereira A.B., Finger I.S., Franz H.C., Torres A.J., Müller V. & Nogueira C.E.W. 2019. **Glucose and insulin curve in pregnant mares and its relationship with clinical and biometric features of newborn foals.** *Pesquisa Veterinária Brasileira 39(9):764-770*. Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão s/n, Cx. Postal 354, Capão do Leão, RS 96010-900, Brazil. E-mail: mmousquer.vet@gmail.com

The aim of the present study was to describe the dynamics of glucose and insulin curves in pregnant mares, and to evaluate the curves according to body condition score, identifying the presence of insulin resistance and correlating these values with the weight, height and clinical changes of the neonates. For this, pregnant mares were evaluated and then grouped according to body condition score during the gestation length until lactation. GrM corresponds to mares with moderate body score (BCS 5-6); GrOv were mares with overweight body score (BCS 7) and GrOb were obese mares (BCS 8-9). A two-step oral sugar test (OST) was used to determine the data. Cortisol analysis was performed with 300-320 days of gestation, at foaling and after parturition. For evaluation of the neonate, a general clinical examination and, weight and height measurements were performed. The results showed hyperglycemia in response to OST with normal insulin values at foaling with a subsequent fall in both values at lactation disregarding group division. Baseline glucose was decreased in GrM compared to GrOv and GrOb with 70-100 days of gestation and with 130-160 days of gestation. With 270-300 days of gestation and post-partum GrOb had increased baseline glucose than GrM. After OST, glucose at foaling day in GrOb presented increased values than GrM. Baseline insulin values did not differ between groups. Post OST insulin levels were higher in GrOb than GrM and GrOv at parturition. No difference in cortisol between moments was identified. GrOb and GrOv maintained increased concentrations after foaling while GrM had a decrease. No correlation was found between maternal glucose and insulin values with foal weight and height, however, a lower ratio between neonatal weight and mare's weight in GrOb and GrOv was identified in relation to the GrM. At foaling, mares presented glucose dysregulation, with obese and overweight mares presenting a greater response to OST.

INDEX TERMS: Glucose, insulin, pregnancy, mares, clinics, biometry, newborn, obesity, overweight, hyperglycemia, foals, horses.

RESUMO.- [**Curvas de glicose e insulina em éguas gestantes e sua relação com as características clínicas e biométricas dos neonatos.**] O objetivo do presente estudo foi descrever a dinâmica das curvas de glicose e insulina em éguas gestantes e avaliar as curvas de acordo com o escore de condição corporal, identificando a presença de resistência insulínica e correlacionando esses valores com o peso, altura e alterações clínicas dos neonatos. Para isso, as éguas prenhes foram avaliadas em conjunto e agrupadas de acordo com o escore

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[2] Departamento de Clínicas Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas (UFPel), Campus Universitário, Capão do Leão s/n, Cx. Postal 354, Capão do Leão, RS 96010900, Brazil. *Corresponding author: mmousquer.vet@gmail.com

[3] Centro Universitário Ritter dos Reis (UniRitter), Av. Manoel Elias 2001, Passo das Pedras, Porto Alegre, RS 91240261, Brazil.

[4] Graduate Studies Program in Animal Medicine: Equine, Universidade Federal do Rio Grande do Sul (UFRGS), Av. Bento Gonçalves 9090, Porto Alegre, RS 90540-000.

de condição corporal durante a gestação até o pós-parto. GrM pertenciam éguas com escore corporal moderado (EC 5-6); GrOv, grupo de éguas com escore corporal acima do peso (EC 7) e GrOb, grupo de éguas obesas (EC 8-9). O teste de glicose oral em duas etapas (OST) foi usado para determinar os dados. A análise do cortisol também foi realizada nos 300-320 dias de gestação, no dia do parto e após o parto. Para avaliação do neonato, foram realizados exame clínico geral e medidas de peso e altura. Os resultados mostraram hiperglicemia em resposta ao OST com valores normais de insulina no momento parto, com uma queda subsequente em ambas as variáveis na lactação, desconsiderando a divisão do grupo. A glicemia basal diminuiu no GrM em comparação com GrOv e GrOb com 70-100 dias de gestação e com 130-160 dias de gestação. Com 270-300 dias de gestação e no pós-parto, o GrOb apresentou aumento na glicemia basal em relação ao GrM. Após OST, a glicose no dia do parto no GrOb apresentou valores aumentados em relação ao GrM. Os valores basais de insulina não diferiram entre os grupos. Após OST níveis de insulina foram maiores no GrOb do que GrM e GrOv no momento do parto. Não houve diferença nos valores de cortisol entre os momentos. O GrOb e GrOv mantiveram cortisol aumentado após o parto enquanto o GrM diminuiu. Não foi encontrada correlação entre os valores de glicemia e insulina materna com o peso e a altura do potro, entretanto, foi identificada uma relação menor entre o peso neonatal e o peso da égua no GrOb e GrOv em relação ao GrM. No parto, as éguas apresentaram desregulação da glicose, sendo que as éguas obesas e com sobrepeso apresentaram uma resposta maior ao OST.

TERMOS DE INDEXAÇÃO: Glicose, insulina, éguas, clínica, biometria, neonatos, gestação, obesidade, sobrepeso, hiperglicemia, equinos.

INTRODUCTION

A progressive decrease in insulin sensitivity is known to occur physiologically in pregnancy. During this period, the mare's metabolism undergoes several changes that help its body to adapt to the placental and fetal growth requirements. The final trimester of gestation is considered the period in which greater mobilization of substrates to the foal occurs, and this period also correlates with increased fetal growth. The mainly source of this substrate is the glucose present in the mare's blood circulation (Ralston 2002, Galantino-Homer & Engiles 2013).

Similarly in woman, Catalano (2010) describes a decrease of at least 50% in insulin sensitivity during gestation. The metabolic syndrome of pregnancy increases the risk of hypertension, nutritional metabolic disorders and inflammation. Although these conditions are solved after childbirth, the woman still faces the risk of developing metabolic syndrome later in life, occurring mainly in individuals who gained considerable weight during pregnancy. Obesity during gestation is also correlated to metabolic alterations, leading to impairment to neonatal health (Catalano et al. 2009). A similar mechanism is thought to occur in equine species (Satterfield et al. 2010).

Obesity is considered a pathological condition, found with increasing frequency in horses and described in several breeds (McCue et al. 2015). This disturbance is related to the development of insulin resistance (IR), known as a reduction in the sensitivity of target tissues to insulin, meaning that in normal concentration, insulin fails to decrease glucose levels, and consequently its concentration remains high in blood causing hyperinsulinemia (Frank & Tadros 2014). IR is involved in the pathophysiology of several chronic endocrine diseases of great importance in equine breeding, which can lead to severe life-threatening consequences (Firshman & Valberg 2007, Frank 2009, 2011). For this reason, obesity can be considered a risk factor during the gestational period.

Fetal development depends on the nutritional, hormonal and metabolic environment provided by the mare, and any change in these variables may reflect in the fetal development and be deleterious to its health in the future (Firshman & Valberg 2007). Mares presenting endocrine changes related to obesity may exacerbate insulin resistance and hyperglycemia, becoming more susceptible to development of laminitis, which in known as the main consequence of hyperinsulinemia in horses, and may potentially lead to abortion (Oke 2013).

There are a few studies evaluating glucose and insulin dynamics during gestation (Ousey et al. 2008, George et al. 2011, Smith et al. 2017, Robles et al. 2018). However, little is known about these dynamics and its relation to the newborn foal. We evaluate insulin resistance throughout the gestational period and, to the authors' knowledge for the first time this evaluation was done at the foaling day. Therefore, the aim of the present study is: i) to describe the dynamics of the glucose, insulin and cortisol curves in pregnant mares and, from these data, ii) to evaluate the curve according to the body condition score during the gestation period, identifying the presence of insulin resistance and its relation to the weight, height and clinical changes of neonates.

MATERIALS AND METHODS

Twenty-six pregnant multiparous pony mares were used, with a mean age of 11 (5-17) years old. The experiment was conducted during the breeding season of 2016. The mares were evaluated by the same trained observer at the beginning of data collection, when they were already confirmed to be pregnant, according to BCS following the system described by Henneke et al. (1983), and then separated and maintained into three distinct groups throughout the entire research period. The animals that had a body condition score (BCS) from 5 to 6 out of 9 were included in the moderate group (GrM, n=5), presenting a mean weight of 355.4 ±19.87kg. Mares identified with a BCS of 7 out of 9 were included in the overweight group (GrOv, n=9), presenting a mean weight of 452±24.65kg, and mares presenting a BCS of greater than 7 out of 9 were considered obese and were included in the obese group (GrOb, n=12) with a mean weight of 523.9±11.47kg. All data collected in this study were approved by the Ethical Committee on Animal of the Federal University of Pelotas, registered under the number CEEA 4579.

In order to evaluate glucose and insulin, a two-step oral sugar test (OST) was performed. The animals remained fasted for 12 hours and, blood was collected the following morning through venipuncture of the external jugular vein in two vacuum tubes (Vacutainer®, U.S.A.) containing sodium fluoride and sodium heparin for glucose and insulin analysis, respectively. At the same time, another blood sample for evaluation of cortisol was collected using a vacuum tube with no anticoagulant. Immediately after the baseline sample collection, corn syrup was administered orally at a dose of 0.15mL/kg, and 60 minutes later the same sample collection was repeated for glucose and insulin analysis.

The oral glucose tolerance test proposed in this study was based on the principle that normally, insulin concentrations should return to basal values after sixty minutes of corn syrup administration. Insulin resistant horses were therefore considered as animals with insulin above 60μUI/mL during that time (Frank 2011). Basal values of these variables above the reference range for equine species were also considered as an indicative of insulin resistance (Frank 2011, Johnson et al. 2010).

Animals were submitted to the two steps OST test at predetermined gestation time points as follows: 70-110 days, 130-160 days, 190-210 days, 270-300 days, 300-320 days, foaling day, 7-10 days postpartum. For cortisol analysis, only 300-320 days, foaling and 7-10 days postpartum were considered. The samples were centrifuged at 800g for 10 minutes; the supernatant was separated and frozen at -20°C until analysis.

Within twelve hours postpartum, the foals were submitted to a general clinical examination, including evaluation of basic parameters such as: heart and respiratory rates, gastrointestinal motility, temperature, mucosal and capillary refill time (CRT). Physical characteristics and presence of alterations were recorded. At that time, weight and height measurements were also performed using a standard weight strip (Corpore foals - Organnact Nutracêuticos®) and a species specific hipometer respectively.

Glucose concentrations were performed using an enzymatic assay (hexokinase method, with commercial kits COBAS Glucose HK c11(catalog number: 04657527; Standard range: 1.98-720mg/dL; Roche Diagnostica Brasil Ltda., São Paulo/SP, Brazil) on a COBAS C11 automatic analyzer, Roche®). Insulin dosages were performed by a chemiluminescent immunoassay using an automatic analyzer (Access immunoassay systems, with commercially available kits - Acess® Ultrasensitive Insulin - catalog number 33410; Standard range: 0.03-300uIU/mL; Intra-assay variation: 2.0-4.2%; Inter-assay variation: 3.1-5.6%, Beckmann Coulter®, Fullerton, CA). For cortisol concentrations, samples were analyzed by B.E.T. laboratories©, Rio de Janeiro/RJ, Brazil, using the radioimmunoassay technique (Cortisol [^{125}I] RIA kit, Standard range: 0-580ng/ml; Intra-assay variation: 5,3-8,9%; Inter-assay variation: 7,5-9,3%, MP Biomedicals LLC, Solon, OH).

The commercial software IBM® SPSS statistics 23 was used for statistical analysis. Descriptive analysis of gestation time, mares' glucose, insulin and cortisol levels, mares' and foals' weight and height are presented as mean ± SE. Firstly, mares' glucose, insulin and cortisol are described with no group separation. Afterwards, they were separated in each corresponding group according to BCS. Data normality was tested using Shapiro Wilk normality test. Non- normally distributed data was transformed to Log10. A one-way repeated measure ANOVA was used. Statistical comparison among groups and group between moments were made by LSD All- Pairwise comparisons, significance was considered at 5% level. A paired t- test was also performed to evaluate difference between glucose and insulin concentrations at baseline and after OST test.

The relationship between the variables weight and height of foals and mares' weight, glucose and insulin concentrations and gestation length was performed using Pearson correlation coefficient. An ANOVA was also performed to determine differences among groups in gestation time, mare's weight, neonatal weight and height and the relation between mare's weight and newborn's weight (M/N) which is described as percent (%). Difference was assessed using LSD All- Pairwise comparison test.

RESULTS

Glucose and insulin curves of all mares during the gestation length, independent of groups, baseline and after 60 minutes of challenge with corn syrup, are shown in the graphs of Figure 1. Baseline glucose and insulin variables remained in the normal reference range in all time points. At foaling, a greater increase in both glucose and insulin values can be observed: 124.8mg/dL and 21.1mIU/mL respectively.

Both baseline and 60 minutes' glucose and insulin concentrations during parturition were different from the remaining moments and a significant decrease in the glucose and insulin values post foaling occurred in all variables. Paired t- test showed a significant increase from basal glucose and insulin values compared to post OST test in al time points in both variables, except in insulin levels from the 130-160 days. No difference was found in cortisol concentration between moments disregarding group separation. Cortisol values in the 5th, 6th and 7th moments were 34.35ng/ml, 41.86ng/ml and 44.78ng/ml respectively.

When the mares were separated into the three distinct groups based on BCS, differences between moments were still

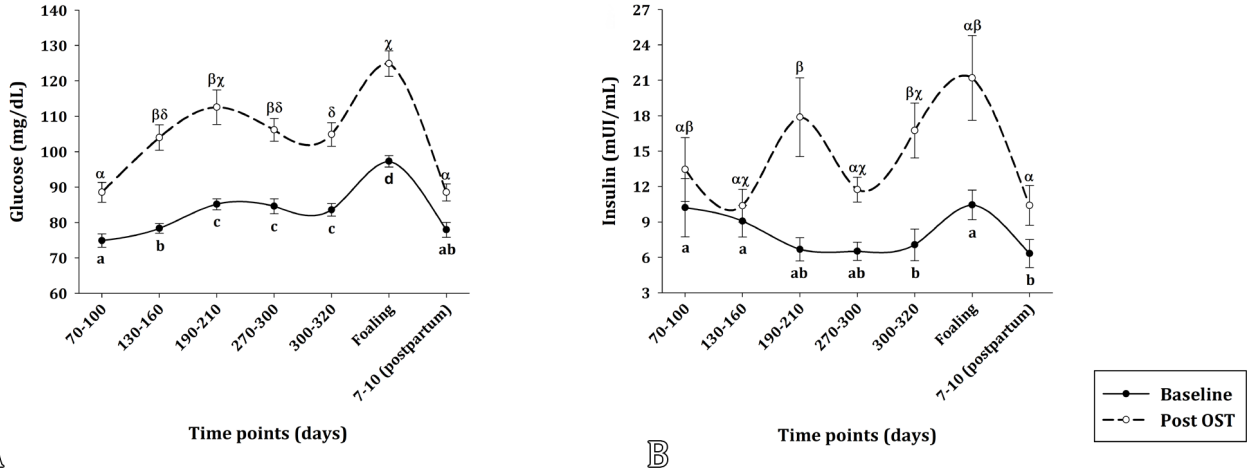


Fig.1. (**A**) Glucose curve of all mares at baseline and after OST during gestation length. (**B**) Insulin curve of all mares at baseline and after OST during gestation length.

observed. Basal glucose curves of the groups are presented in Figure 2A. Considering the variations between groups in each time point, fasting glucose was lower in GrM compared to GrOv and GrOb at 70-100 days and 130-160 days. At 270-300 days and 7-10 days after foaling moments, GrOb had higher basal glucose levels when compared with GrM, but no difference was found from GrOv.

After corn syrup administration, an increased glucose response is seen at the day of foaling with a subsequent fall after parturition (Fig.2B). Regarding group comparison, at 70-120 days of gestation, GrM presented an increased response when compared with GrOb. The response during time points 130-160 days, 190-210 days, 270-300 days, and 300- 320 days remained constant in all three groups, and, at foaling, a raise in glucose levels in GrOb compared to GrM was observed, with no difference from GrOv.

Resting insulin concentrations remained within normal reference range in all time points (Fig.2C). No difference was found between groups in any time points. After glucose administration, insulin levels were increased in GrOb when compared to GrM and GrOv at foaling (Fig.2D). No differences were observed in the remaining time points. Regarding cortisol analysis, no difference between time points were identified, however, GrOb and GrOv maintained increased concentrations after foaling while GrM had a decrease (Fig.3). No difference between groups in cortisol was found in the remaining time points.

All the foals presented normal clinical findings in all groups. Regarding the physical features, only foals from GrOv and GrOb presented mild alterations. Changes like angular deviations (1/21), flexural deformities including hypertension and hyperflexion of flexor tendons in hind and front limbs (7/21) and bulging of the frontal bone in the head (6/21) were observed.

Newborns' weight, height and mares' gestation length were not different between groups. However, mares of GrOb had higher weight, followed by GrOv and then GrM. In addition, mares from GrOb presented a smaller relation between maternal and neonatal weight (Table 1). At Pearson's correlation test, no correlation between newborn weight and

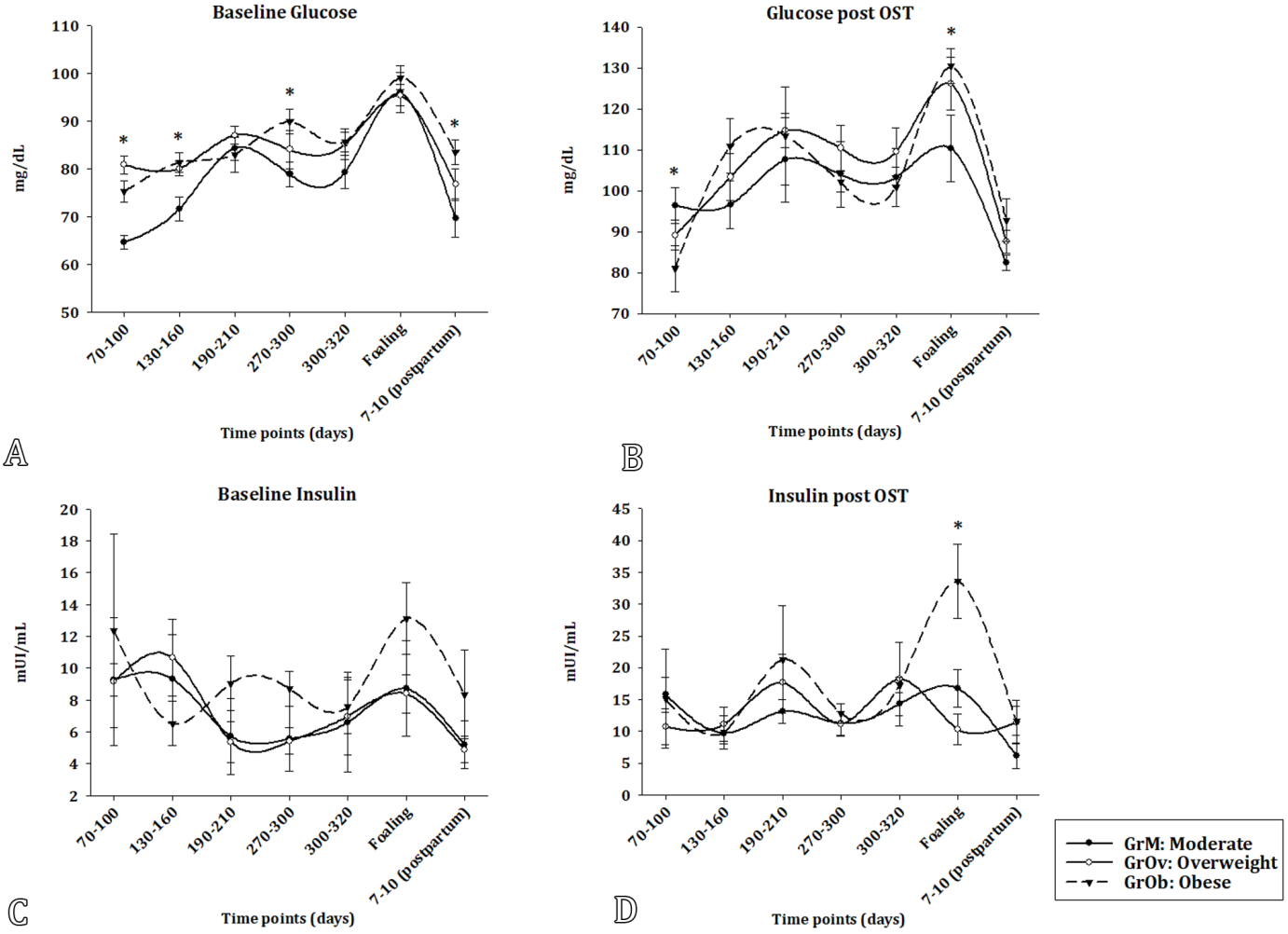


Fig.2. Baseline and post OST glucose and insulin curves of mares separated in the three groups according to body condition score. (**A**) Baseline glucose levels during gestation length in moderate group (GrM), overweight group (GrOv) and obese group (GrOb). (**B**) Glucose concentration after 60 minutes of OST in moderate group (GrM), overweight group (GrOv) and obese group (GrOb). (**C**) Baseline insulin concentration during gestation in moderate group (GrM), overweight group (GrOv) and obese group (GrOb). (**D**) Insulin concentration 60 minutes after OST during gestation in moderate group (GrM), overweight group (GrOv) and obese group (GrOb).

Table 1. Descriptive values of mare's gestational length, weight, neonatal weight and height and the relation between maternal weight and foal birthweight in moderate group (GrM), overweight group (GrOv) and obese group (GrOb)

Group	Gestation length(days)	Mare weight (Kg)	Neonatal weight (Kg)	Neonatal height (cm)	M/N relation (%)
GrM	336,8 ± 3,3	355,4 ± 19,87[a]	38,20 ± 1,46	87,86 ± 1,14	10,54[a]
GrOv	334,3 ± 2,7	452,0 ± 24,65[b]	41,75 ± 2,32	92,71 ± 0,84	9,25[b]
GrOb	330,9 ± 1,6	523,9 ± 11,47[c]	44,36 ± 1,95	92,00 ± 1,00	8,49[b]

Results are shown as mean±SE; [a,b,c] different letters indicate statistical difference between groups (P<0,05); M/N relation = indicates maternal and newborn's weight relation, which is shown as percent.

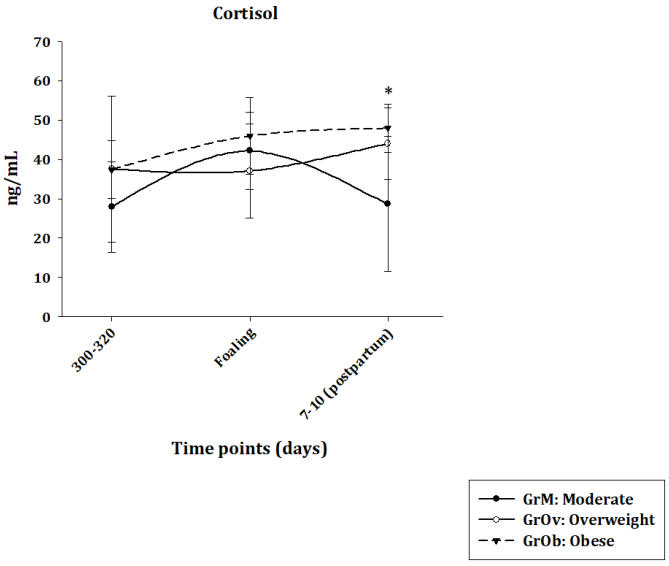


Fig.3. Cortisol concentration at 300-320 days of gestation, at the foaling day and postpartum in moderate group (GrM), overweight group (GrOv) and obese group (GrOb).

height with mare's glucose and insulin concentration was found. However, mares' weight correlated with newborn weight (r=0.640, p=0.02) and height (r=0.610, p=0.02), gestation length correlated negatively with newborns' weight (r= -0.435, p= 0.03) and the relation among mares' and foal' weight correlated negatively with mares' baseline insulin concentration at foaling (r= -0.42, p=0.04).

DISCUSSION

None of the mares showed basal hyperglycemia across all time points, once these values were within the reference range (Frank 2011, Frank & Tadros 2014). However, the majority of the mares showed hyperglycemia (>115mg/dL) after OST challenge at the moment of foaling with three of them also presenting increased insulin concentration (60µUI/mL) at that time, suggestive of insulin resistance (Johnson et al. 2010). Increased glucose response can occur due to a primary inadequacy of β-cell secretion of insulin, impaired disposal of glucose, or fat adaptation that spares glucose utilization without decreasing insulin sensitivity (Kronfeld et al. 2005).

When lower peaks of insulin with higher glucose concentrations are identified it is referred as uncompensated IR, which indicates pancreatic insufficiency or beta cell exhaustion, resembling what occurs in diabetes mellitus (Frank 2009, Frank & Tadros 2014). This can be identified in horses with pituitary pars intermedia dysfunction (PPID), although, none of the mares of this study presented clinical signs compatible with the disease (Frank 2009, Frank & Tadros 2014). Decompensation was also identified in a similar diabetic- like condition occurring during pregnancy (Trieber 2006), which is more likely to have occurred in these mares at the time of foaling. When glucose and insulin are both elevated it's considered a transient state between compensated to uncompensated IR (Trieber 2006).

It should be considered that insulin and glucose peaks have been identified in other studies after sixty minutes the OST test, which could have interfered in insulin resistance analysis in the current study, as evaluation was done in just one time point after OST due to the study being conducted on a private farm, where stress was managed during the procedures to avoid pregnancy loss (Hoffman et al. 2003, Bamford et al. 2014). Besides that, to the author's knowledge, it is the first time that increased response peaks of insulin and glucose are observed at the day of foaling in equine species.

Compensated IR was not identified in the remaining moments before parturition as described by literature (Fowden et al. 1984, George et al. 2011), probably due to reasons previously mentioned. However, from 190-210 days insulin response after oral glucose syrup was higher from basal values, which are in the time frame considered with most glucose uptake for fetal placental growth which is in agreement with previous studies (Fowden et al. 1984, Galantino-Homer & Engiles 2013). This increase in insulin response to glucose is mediated by insulin synthesis and secretion. During pregnancy, it occurs as an adaptive mechanism that slows clearance of glucose from maternal tissues to ensure sufficient glucose supply for fetoplacental tissues (George et al. 2011). The maternal alteration in glucose and insulin dynamics during pregnancy is also correlated with actions of other hormones connected to gestation (Galantino-Homer & Engiles 2013).

At lactation, glucose and insulin concentrations and response returned to normal, which is in agreement with other study (Bryden et al. 2013). In this case, a lower concentration and a faster removal of glucose after an oral test with lower peak occur due to redirection of glucose to the mammary gland for milk production (Firshman & Valberg 2007).

Cortisol concentrations of all mares did not show any differences between time points. Similar serum cortisol values in pregnant mares have been previously described (Flisinska-Bojanowka et al. 1992, Satué et al. 2011, Marcilla et al. 2017). In pregnancy, this hormone is involved in the regulation of fetal metabolism and growth (Marcilla et al. 2017). Cortisol is also a counterregulatory hormone of glucose, antagonizing the effects of insulin and promoting hepatic gluconeogenesis and inhibition of cellular glucose uptake (Firshman & Valberg

2007, Hart et al. 2016). Nagel et al. (2012) describes an increase in plasmatic cortisol levels in the fourth days prior to parturition, with even higher concentration occurring at the day of foaling to at least 8 hours after. Other studies have demonstrated that the greater increase in cortisol values during pregnancy occurs between the fourth and fifth months, decreasing progressively in the following period, which is suggested to be negatively correlated with estrogens concentrations as the gestation progresses (Satué et al. 2011, Marcilla et al. 2017).

Considering group evaluations, the higher glucose and insulin response in the obese and overweight mares, especially at foaling, suggests that they could exacerbate glucose and insulin dysregulation at that time point, as was observed in this study (Morresey 2012). Metabolic dysregulation was associated with obesity in pregnant mares and women in other studies (George et al. 2009, Catalano 2010, Peugnet et al. 2015). Conversely, Song (2014) found insulin dysregulation in mares with low BCS due to β- pancreatic cell deficiency.

When groups were evaluated for cortisol concentrations, obese and overweight mares maintained high cortisol levels within 7-10 days after foaling while moderate BCS mares had a significant decrease at that moment. Cortisol demand is closely related to the degree of metabolic activity, which is increased in the initial lactation period due to glucose requirement for milk production and could also be related to high BCS. Together with other related hormones, cortisol remains high because it is responsible for maintaining milk production (Satué et al. 2011).

Although no correlation was found between maternal insulin and glucose levels with the weight and height of the neonate, the present data show that in the group of obese mares, maternal weight and foal birth weight relation of 8.49% was obtained, and the overweight group had a relation of 9.25%, being significantly lower than the group with a moderate BCS ($P<0.05$). According to Brown-Douglas et. al. (2011), the weight of the neonate should correspond to 10% of the dam's body weight, which is considered a reflection of the placental efficiency. Smith et al. (2017) have identified an inverse relationship between body score and foal weight at birth than what we found in the present study, observing that mares with higher body scores gave birth to heavier foals. It was also observed that the majority of physical alterations presented by foals of GrOv and GrOb at birth were consistent with immaturity (Stoneham 2006).

The relation of maternal weight and foals' birthweight correlated negatively with mare's fasting insulin at parturition, showing that mares with higher insulin concentrations at foaling had lower weight percentage relation between mare and foal. However, this data should be further investigated.

CONCLUSIONS

At foaling, mares presented glucose and insulin dysregulation. During earlier gestational period, it also showed diminished glucose removal rate, which is related to fetal-placental growth demand. Obese and overweight mares had greater response to OST at the foaling day and higher cortisol values in post-partum.

Insulin resistance was not identified in the majority of the mares. It was not observed difference in weight and height of the newborns in the different groups, however, obese and overweight mares presented a lower relation between maternal weight and foal birthweight. No correlation of newborn weight and height with mare's glucose and insulin values were found.

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Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

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

- Livros:

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

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

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

- Capítulos de livros:

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

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

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- Dissertações e Teses:

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

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

- Resumos publicados em eventos:

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

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

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

Colégio Brasileiro de Patologia Animal

Em 15 de agosto de 2019

GUIDE FOR AUTHORS

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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Exemples for References:

➢ Articles published in scientific journals:

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

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

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

➢ Books:

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

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

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

➢ Chapters of books:

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

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

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

➢ Dissertations and Theses:

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

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

➢ Abstracts published in Events:

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

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

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

(Note: Consult entire papers instead of only Abstracts)

Pesquisa Veterinária Brasileira

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