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Cover illustration: Enlarged and yellowish liver of sheep with pregnancy toxemia. (Souto et al., p.784)

Techniques for prevention and control of poisoning by sodium monofluoroacetate (MFA)-containing plants in ruminants¹

Danielle A.N. Pessoa^{2*} , Rosane M.T. Medeiros³ and Franklin Riet-Correa⁴ 

ABSTRACT.- Pessoa D.A.N., Medeiros R.M.T. & Riet-Correa F. 2019. **Techniques for prevention and control of poisoning by sodium monofluoroacetate (MFA)-containing plants in ruminants.** *Pesquisa Veterinária Brasileira* 39(10):771-779. Centro de Saúde e Tecnologia Rural, Hospital Veterinário, Universidade Federal de Campina Grande, Avenida Universitária s/n, Bairro Santa Cecília, Patos, PB 58780-110, Brazil. E-mail: danipessoavet14@gmail.com

Monofluoroacetate (MFA)-containing plants cause sudden death associated with exercise in ruminants, and are responsible for significant losses in Brazilian livestock, estimated at 500,000 bovine deaths annually. Most of the times, the control and treatment of this type of poisoning are not efficient, because disease evolution is superacute, usually causing the death of the animal. Due to the difficulty in controlling this intoxication, several studies have suggested alternatives to prevent it, mainly by making animals resistant to the MFA present in these plants or by avoiding their consumption. This literature review addresses the techniques used experimentally to control the poisoning of ruminants by plants containing MFA. The first studies carried out in Brazil demonstrated that goats and sheep that continuously receive non-toxic doses of plant containing MFA show greater resistance to poisoning than untreated animals, and that this resistance can be transmitted by ruminal fluid transfaunation, suggesting that poisoning occurs due to the presence of bacteria that hydrolyze MFA in the rumen. Based on this hypothesis, several MFA-hydrolyzing bacteria were isolated (*Enterococcus faecalis*, *Bacillus* sp., *Paenibacillus* sp., *Burkholderia* sp., *Cupriavidus* sp., *Staphylococcus* sp., *Ancylobacter* sp., *Ralstonia* sp., *Stenotrophomonas* sp., *Pigmentiphaga kullae*, and *Ancylobacter dichloromethanicus*). When some of these bacteria were administered intraruminally, they provided the animal with a different level of protection against poisoning. However, it was observed that protection is gradually lost when the bacterium administration is interrupted. Consequently, to obtain more efficient protection, these bacteria should be administered continuously, probably in the form of probiotics. In another assay, MFA was administered to sheep at non-toxic doses to test the hypothesis that this substance could induce the multiplication of bacteria that hydrolyze it in the rumen. There was no increase in resistance to poisoning after administration of MFA; however, no signs of poisoning were observed when animals received trifluoroacetate and no clinical signs were verified when they were challenged with toxic doses of MFA; in contrast, all control animals presented clinical signs. These results suggest that trifluoroacetate induces the proliferation of MFA-degrading bacteria, and can be used in intoxication prophylaxis. The conditioned food aversion technique, using lithium chloride, has been successfully used experimentally to prevent ruminants from ingesting plants that contain MFA. Another alternative tested was the spraying of *Amorimia septentrionalis* with the endophytic bacterium *Herbaspirillum seropedicae*, which degrades MFA, resulting in decreased concentration of this compound

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in the plants. In conclusion, several experimental techniques have been proved efficient in the control and prophylaxis of MFA-containing plant poisoning; however, none of these techniques are available commercially. Further experiments, mainly in the field, should be carried out to adapt some of these techniques to the conditions of extensive breeding in the numerous areas where MFA-containing plants occur.

INDEX TERMS: Prevention, control, poisoning, ruminants, sodium monofluoroacetate, toxic plants, MFA-containing plants, sudden death, poisoning control, sheep, goats, cattle, plant poisoning.

RESUMO.- [Técnicas para prevenção e controle da intoxicação em ruminantes por plantas que contém monofluoroacetato de sódio.] As plantas que contém monofluoroacetato (MFA) causam morte súbita associada ao exercício em ruminantes, e são responsáveis por grandes perdas na pecuária brasileira, estimadas em 500.000 mortes de bovinos anualmente. O controle e tratamento desse tipo de intoxicação, na maioria das vezes, não apresenta eficiência, visto que a evolução da doença é superaguda, e geralmente ocasiona a morte do animal. Devido à dificuldade no controle dessa intoxicação, diversos estudos sugerem alternativas para preveni-la, principalmente tornando os animais resistentes ao MFA presente nessas plantas ou evitando seu consumo. O objetivo do presente trabalho é fazer uma revisão bibliográfica das técnicas utilizadas experimentalmente para controlar a intoxicação de ruminantes por plantas que contém MFA. Nos primeiros trabalhos realizados no Brasil, foi determinado que caprinos e ovinos que recebem continuamente doses não tóxicas de planta que contém MFA apresentam maior resistência a intoxicação que animais não tratados e que essa resistência pode ser transmitida por transfaunação de fluído ruminal, sugerindo que a mesma ocorre devido a presença de bactérias que hidrolisam MFA no rúmen. Com base nessa hipótese foram isoladas diversas bactérias que hidrolisam MFA (*Enterococcus faecalis*, *Bacillus* sp., *Paenibacillus* sp., *Burkholderia* sp., *Cupriavidus* sp., *Staphylococcus* sp., *Ancyllobacter* sp., *Ralstonia* sp., *Stenotrophomonas* sp., *Pigmentiphaga kullae* e *Ancyllobacter dichloromethanicus*). Quando algumas dessas bactérias foram administradas intraruminalmente conferiram diferentes graus de proteção contra a intoxicação. No entanto foi observado que a proteção se perde gradualmente quando se deixa de administrar a(s) bactéria(s). Em consequência, para obter uma proteção mais eficiente essas bactérias deveriam ser administradas continuamente, provavelmente na forma de probiótico. Em outro ensaio administrou-se MFA a ovinos em doses não tóxicas para testar a hipótese de que esta substância poderia induzir a multiplicação de bactérias que hidrolisam o mesmo no rúmen. Não houve um aumento da resistência a intoxicação após a administração de MFA; no entanto quando foi administrado trifluoroacetato, os animais não desenvolveram nenhum sinal de intoxicação e quando desafiados com doses tóxicas de MFA não apresentaram sinais clínicos, pelo contrário todos os animais controles apresentaram sinais clínicos. Esses resultados sugerem que o trifluoroacetato induz a proliferação de bactérias que degradam MFA e pode ser utilizado para a profilaxia da intoxicação. A técnica da aversão alimentar condicionada, utilizando cloreto de lítio, tem sido empregada experimentalmente, com sucesso, para evitar que ruminantes ingiram plantas que contém MFA. Outra alternativa testada foi a pulverização de *Amorimia septentrionalis* com a bactéria endofítica *Herbaspirillum seropedicae*, que degrada MFA, resultando na diminuição da

concentração deste composto na planta. Conclui-se que há diversas técnicas que experimentalmente tem demonstrado eficiência no controle e profilaxia das intoxicações por plantas que contém MFA; no entanto, nenhuma dessas técnicas está disponível comercialmente. Futuros experimentos, principalmente, a campo, deverão ser realizados para adaptar alguma(s) dessas técnicas as condições de criação extensiva nas numerosas áreas onde ocorrem plantas que contém MFA.

TERMOS DE INDEXAÇÃO: Prevenção, controle, intoxicação por plantas, ruminantes, monofluoroacetato de sódio, plantas tóxicas, plantas que contém MFA, morte súbita, controle de intoxicações, ovinos, caprinos, bovinos.

INTRODUCTION

Currently, there are 131 toxic plant species, divided into 79 genera, in Brazil. Among these, the group of toxic plants that cause exercise-related sudden death is represented by 22 species belonging to three families: Rubiaceae, Malpighiaceae, and Bignoniaceae (Nascimento et al. 2018). The presence of sodium monofluoroacetate (MFA) as a toxic principle has already been confirmed in the following species: *Palicourea marcgravii*, *Palicourea aeneofusca* (Lee et al. 2012), *Palicourea grandiflora*, *Palicourea aff. juruana*, *Palicourea amapaensis*, *Palicourea macarthurorum*, *Palicourea nigricans*, *Palicourea vacillans*, *Palicourea barraensis* (formerly *Palicourea aff. longiflora*), *Palicourea longiflora* (Cook et al. 2014, Carvalho et al. 2016), *Amorimia amazonica*, *Amorimia exotropa*, *Amorimia pubiflora*, *Amorimia rigida*, *Amorimia septentrionalis* (Lee et al. 2012), *Niedenzuella stannea* (Arruda et al. 2017), and *Tanaecium bilabiatum* (formerly *Arrabidaea bilabiata*), misidentified as *Fridericia japurensis* (*Arrabidaea japurensis*) in the state of Roraima, Brazil (Lima et al. 2016).

Plants that cause sudden death in ruminants are important throughout the country, as poisoning generally triggers the death of animals, causing irreparable losses to the herd. Cattle are more sensitive to these plants than goats and sheep, so much so that approximately 500,000 cattle annual deaths from toxic plant poisoning are caused by exercise-related sudden death (Pessoa et al. 2013).

Among the plants that cause sudden death and contain MFA, the most important and responsible for outbreaks of ruminant poisoning in Brazil are *P. marcgravii*, found in practically all Brazilian territory, except for the southern, semi-arid and northeastern states, and Mato Grosso do Sul state (Tokarnia et al. 2012); *P. aeneofusca*, which is present in the states of Paraíba, Pernambuco, Alagoas and Bahia (Brito et al. 2016); *A. septentrionalis*, formerly *Mascagnia rigida* (Davis & Anderson 2010), found mainly in the caatinga, on rocky outcrops, in the states of Pernambuco, Alagoas, Rio Grande do Norte, Paraíba and Ceará. *A. septentrionalis* is

considered the most important toxic plant for the northeastern semiarid region (Duarte et al. 2013). In northern Brazil, the most important species are *P. grandiflora*, found in the states of Acre, Amazonas, Pará and Rondônia; *P. barraensis*, present in the states of Amazonas, Roraima and Rondônia; *P. longiflora*, found in the states of Amazonas, Pará and Rondônia; *Amorimia amazonica* present in the states of Acre, Amazonas and Rondônia (Duarte et al. 2013, Cook et al. 2014, Carvalho et al. 2016), and *T. bilabiatum*, which can be found throughout the Brazilian territory, but is the most important toxic plant for cattle after *P. marcgravii* in the Amazon region (Tokarnia et al. 2012).

There are marked variations in the amount of MFA both between plant parts and between plants belonging to the same genus, same species and, consequently, between plants of different genera (Lee et al. 2012). Plants of genus *Palicourea* have more significant amounts of this compound than those of the other genera; they can contain 50 to 100 times more MFA than those of the genus *Amorimia* (Lee et al. 2012). In *P. marcgravii*, the amounts of MFA found were 0.88% in leaves at the beginning of development and 0.24% in mature leaves, whereas in *P. aeneofusca*, the concentration in leaves was 0.09% (Lee et al. 2012). Variation in the amount of MFA may occur even between plants of the same species, for example, MFA concentration ranged from 0.03 to 0.58% in *P. marcgravii* and from 0.03 to 0.18% in *P. aeneofusca* (Cook et al. 2014). In *P. longiflora*, MFA leaf content ranged from 0.006 to 0.16%, and in the leaves of *P. barraensis*, the concentration was 0.01% (Carvalho et al. 2016).

MFA leaf contents in *A. amazonica*, *A. exotropa*, *A. rigida* and *A. pubiflora* were 0.0007, 0.02, 0.002 (Lee et al. 2012) and 0.22% (Lima et al. 2019), respectively. In *A. septentrionalis*, collected in Paraíba state, the concentrations found varied according to plant part: 0.002% in mature leaves, 0.001% in stems, 0.008% in flowers, and 0.006% in seeds (Lee et al. 2012). In *A. septentrionalis* samples collected in Pernambuco state, the MFA concentration was practically the same (0.0021%) in the leaves of the adult plant (Albuquerque et al. 2014). However, Lopes et al. (2019) found 0.00074% MFA in the leaves of *A. septentrionalis* and Pessoa et al. (2019, unpublished data) found 0.16% MFA also in *A. septentrionalis* leaves; the two plants collected in the same location in Paraíba state. This variation reinforces the claims that plant toxicity varies according to developmental stage (Lee et al. 2012, Tokarnia et al. 2012), since plants collected in the same region showed variations in toxicity. Although these studies do not mention the time of sample collection, these plants were probably collected at different times of the year.

In *N. stannea*, MFA concentrations in leaves were lower (0.0003%) when compared with those of seeds (0.06%), fruits (0.0008 to 0.02%), and flowers (0.0003 to 0.006%) (Arruda et al. 2017); and in *T. bilabiatum*, MFA leaf content ranged from 0.0001 to 0.041% according to plant sample collection location (Lima et al. 2016).

MFA is a highly toxic compound for all animal species, including humans. It acts on the tricarboxylic acid cycle (Krebs Cycle) through the so-called "lethal synthesis", in which the active metabolic (fluorocitrate) blocks the action of coenzymes, precursors for the formation of ATP, consequently blocking metabolic processes dependent on energy and the

accumulation of citrate in the tissues, leading to the death of the animal (Nogueira et al. 2011).

The main clinical signs usually associated with exercise observed in poisoning by MFA-containing plants in ruminants are apathy, anorexia, tachycardia, tachypnea, reluctance to move, vocalization, diarrhea, muscle tremors, jugular venous engorgement, motor incoordination, sternal decubitus evolving to lateral decubitus, pedaling movements, opisthotonus and falling (Vasconcelos et al. 2008, Oliveira et al. 2013, Pessoa et al. 2015).

Treating this type of poisoning is a major challenge for veterinarians, as evolution is superacute, most often resulting in the death of animals. Some conventional measures to control poisoning, such as herbicide use, isolation of infested areas and manual removal of plants, are not efficient, especially in properties with large pasture areas and extensive farming (Riet-Correa & Medeiros 2001, Pessoa et al. 2013). Tokarnia et al. (2012) suggested avoiding moving the animals for 7-15 days; it is believed that this is the average time required for MFA to be detoxified from the organism. Due to this difficulty in the treatment and prevention of this type of intoxication, several studies have been conducted in order to make the animals resistant to poisoning, and later develop techniques that can be used in areas where plants containing MFA are common. This study aimed to review the control methods for poisoning by MFA-containing plants in ruminants.

Administration of non-toxic doses of MFA or MFA-containing plants

In Brazil, there are widespread reports that animals coming from areas with occurrence of MFA-containing plants are more resistant to poisoning by plants containing this compound than animals grazing on pastures of free of these plants (Silva et al. 2008). It is believed that this occurs because of the time of evolutionary exposure to these pastures, by dietary preference, levels of dietary specialization, and habitat preference (Twigg & King 2000), as well as because these animals possibly have bacteria in their rumen that are capable of degrading the MFA through production of the fluoroacetate dehalogenase enzyme (Becker et al. 2016).

Several studies have been performed with the objective of proving that daily administration of non-toxic doses of MFA-containing plants provides resistance to poisoning by plants containing this compound in ruminants.

Oliveira et al. (2013) initially offered six goats (Group 1) successive non-toxic doses of *Palicourea aeneofusca* in alternating periods: 0.02g/kg for two periods of five days and 0.03g/kg for two five-day periods, with intervals without ingesting the plant of 10 days between the first and second periods and of 15 days between the third and fourth periods. Fifteen days after the last administration period, these goats, along with six other goats (Group 2) that did not receive previous doses of the plant, were challenged with 0.03g/kg of the plant for 19 days, and as of the 20th day, the dose was increased to 0.04g/kg and administered for further 12 days. After these 31 days of administration, five goats in Group 1 and three goats in Group 2 remained without clinical signs of poisoning. Subsequently, to verify whether these animals remained resistant, three additional goats (Group 3) were added to the experiment. As of day 32 of the experiment, these eight remaining animals, along with the three recently

added ones, were challenged with a dose of 0.06g/kg of the plant until the 40th day. In Group 1, five animals remained resistant and without clinical signs of poisoning during the whole experiment (40 days of challenge). In Group 2, three animals showed no clinical signs of poisoning after ingesting the plant for 12 days at a daily dose of 0.03g/kg and for another 20 days at a dose of 0.04g/kg. In Group 3, all animals that did not receive prior administration of doses became ill three days after ingestion of 0.06g/kg. These results showed that the administration of non-toxic doses of *P. aeneofusca* induces resistance to poisoning by this plant in goats.

Duarte et al. (2014) administered six goats increasing non-toxic doses of *Amorimia septentrionalis* in alternating periods: 1g/kg for 10 days, 2g/kg for two periods of five days, and 3g/kg for two five-day periods, always with intervals of 15 days without ingesting the plant between the administration periods. After the last administration period, the goats were given 3g/kg of the plant for seven days to verify their resistance to poisoning. As control, six other goats received the same dose of 3g/kg daily, without previous doses, and were considered untreated animals. Duarte et al. found that the goats that received previous non-toxic doses (considered treated) did not show clinical signs of poisoning during the whole plant administration period (challenge). Untreated animals showed clinical signs as of the 4th day of administration, and two deaths were recorded. Santos et al. (2014) provided sheep with increasing non-toxic doses of MFA to assess whether exposure to this compound would make animals resistant to it. The sheep were divided into two groups. In Group 1, the animals received non-toxic, increasing doses of MFA for six periods: 0.05mg/kg for five days, 0.08mg/kg for two periods of four days, 0.1mg/kg for two three-day periods, and 0.25mg/kg for three days. Between the first three administration periods, the animals did not receive MFA for 10 consecutive days; between the other administration periods, the sheep remained 15 days without ingesting the MFA. In Group 2, the sheep did not receive previous doses of MFA and served as controls. After this resistance induction period, all sheep were challenged with a single dose of 1mg/kg MFA. Duarte et al. found that the administration of non-toxic doses of MFA does not induce resistance to this compound as experimental animals showed clinical signs of poisoning, and some even died, probably because absorption of the pure compound by the organism would be faster than when it is present in the plants, which facilitates its degradation.

Trifluoroacetate is a chemical compound formed by the breakdown of various hydrofluorocarbons and hydrochlorofluorocarbons (Cahill et al. 2001), and its use in the treatment and control of poisoning by MFA-containing plants in ruminant has been previously studied. Costa et al. (2019) administered a group of calves 0.1mg/kg of sodium trifluoroacetate dissolved in 5mL of water, and another group of calves received only water. This administration was performed orally, using a syringe placed at the base of the tongue, daily for 28 days. After this administration period, calves in both groups received 2g/kg of fresh leaves of *P. marCGravii* through an orogastric tube, and were then stimulated to move in order to trigger the clinical condition of poisoning. Costa et al. (2019) observed, by clinical examination and blood tests, that the compound did not cause any toxic

effects on the animals throughout the administration period. The animals that received sodium trifluoroacetate did not show any clinical signs of poisoning after administration of the plant, whereas those that did not receive the compound showed clinical signs of poisoning. Costa et al. (2019) concluded that sodium trifluoroacetate induces resistance to poisoning by MFA-containing plants probably because this compound promotes multiplication of MFA-hydrolyzing bacteria.

Becker et al. (2016) administered four sheep non-toxic doses of *A. pubiflora* in alternating periods: 0.5g/kg for 20 days and, after a 15-day interval, 1g/kg for three days. Another four sheep served as controls, and received only 1g/kg of the plant for three days. It took 89h27min $\pm$ 15h32min for the sheep that received the non-toxic doses to develop clinical signs of poisoning, whereas the control sheep developed signs after 23h15min $\pm$ 00h37min, and all signs progressed to death. It took longer for the animals that received previous non-toxic doses of the plant to develop clinical signs of poisoning, and they did not die, proving resistance induction after ingestion of these doses.

Use of rumen transfaunation to transfer resistance to poisoning by MFA-containing plants

Transfaunation consists in the transfer of ruminal fluid from a healthy to an unhealthy ruminant in order to reestablish the ruminal flora of the sick animal. It is a widely performed procedure in the practice with ruminants, and is generally indicated in cases of indigestion or whenever the ruminal microbiota is compromised or with reduced activity (Radostits et al. 2007, Lira et al. 2013). Some studies have found that it is possible to transfer resistance to poisoning by MFA-containing plants by transfaunation from resistant animals to susceptible animals. Possibly, this occurs because transfaunation promotes changes in the rumen flora probably resulting from proliferation of microorganisms capable of degrading MFA.

Duarte et al. (2014) performed transfaunation from goats considered resistant to *Amorimia septentrionalis* poisoning, after administration of non-toxic doses of the plant, to susceptible goats grazing in areas free from occurrence of MFA-containing plants. After transfaunation, the plant was offered to the animals to verify whether resistance was transferred. They found that it took 19 $\pm$ 7.5 days for goats that received ruminal fluid to develop clinical signs of *A. septentrionalis* poisoning, whereas it took 7.8 $\pm$ 1.9 days in the case of goats that did not undergo transfaunation. The goats that underwent transfaunation were later found to be more resistant to poisoning than the controls.

Silva et al. (2015) carried out rumen transfaunation from goats considered resistant to poisoning by *A. septentrionalis*, after oral administration of *Ancylobacter dichloromethanicus* and *Pigmentiphaga kullae* bacteria, to poisoning-sensitive goats, that is, animals that had never had contact with MFA-containing plants. After the transfaunation, *A. septentrionalis* was administered at a dose of 5g/kg to a group of goats, whereas the other goats received only the plant as controls. Silva et al. found that the animals that received ruminal fluid did not develop clinical signs of poisoning and did not die after administration of *A. septentrionalis* for eight days; however, all those who did not undergo transfaunation developed severe clinical signs as of day 2 of plant ingestion, and in one animal these signs evolved to death.

In 2016, a group of researchers transferred ruminal fluid from sheep that were resistant to *A. pubiflora* poisoning, following successive administration of non-toxic doses of the plant, to sensitive sheep in order to assess whether poisoning resistance was also transferred. They found that the sheep that received ruminal fluid were resistant to poisoning, being able to ingest larger amounts of the plant and develop clinical signs of poisoning much later (Becker et al. 2016).

The aforementioned experiments demonstrated that both the administration of non-toxic doses of plants and rumen transfaunation from resistant to sensitive animals are effective to induce resistance to poisoning by MFA-containing plants probably because both techniques favor the presence and multiplication of bacteria that degrade this compound in the animal rumen (Oliveira et al. 2013, Duarte et al. 2014, Becker et al. 2016). Nevertheless, it is not possible to use these techniques in commercial breeding, mainly because of the difficulty in standardizing the plant dose used, as there are marked variations in plant toxicity. Further studies addressing the identification of MFA-hydrolyzing bacteria and the use these bacteria to control plant poisoning are needed.

Oral administration of MFA-degrading bacteria

The use of MFA-degrading bacteria in the control of poisoning by plants containing MFA in ruminants has been addressed in several studies because they are bacteria capable of using MFA as carbon source and could adapt in environments such as the rumen. The bacteria capable of breaking the strong bond between carbon and fluorine in MFA are believed to degrade this compound because they produce enzymes called dehalogenases that can catalyze the reaction, causing these bacteria to use this compound as a source of carbon and energy (Fetzner & Lingers 1994, Firsova et al. 2009). Gregg et al. (1998) orally offered sheep the ruminal bacterium *Butyrivibrio fibrisolvens*, genetically modified from a plasmid (pBHf) of a *Moraxella* species. They then challenged these animals with different doses of fluoroacetate and found that the sheep that received the bacterium were resistant to higher doses of fluoroacetate, proving the protective effect of the bacterium. Padmanabha et al. (2004) used the same bacterium as Gregg et al. (1998) in cattle to test their ability to protect against MFA poisoning. After challenge with the compound, they observed that the cattle that received the bacterium presented a reduction of the toxic effects of MFA in their organisms, and became resistant to higher doses of it.

The use of genetically modified bacteria in countries such as Australia, for instance, has not been approved owing to strict governmental laws that prevent the use of transgenic organisms in several areas (Leong et al. 2017). There was also concern about the use of these bacteria because, in Australia and New Zealand, MFA is widely used to control vertebrate animals (rabbits, foxes, skunks, dingoes, etc.), which are considered pests in agricultural production; in addition to the fear of these bacteria being eliminated in the environment and colonizing the organisms of these animals, making them also resistant to MFA and difficult to control (Twigg & King 2000).

Pimentel (2011) isolated *Enterococcus faecalis* and *Bacillus* sp. from the ruminal content of cattle and found that these bacteria were capable of degrading MFA, suggesting their use in the control of poisoning by both plants containing MFA and by the compound itself. Dias (2015) tested the ability of

Enterococcus faecalis to induce resistance to MFA poisoning in sheep. The bacterium was inoculated into the rumen of the sheep using a 10ml rumen tube for three days. Other sheep received water instead of bacteria and were considered as controls. Forty-eight hours after the last inoculation, 1.5mg/kg MFA was given to all animals orally in a single dose. Pimentel concluded that *Enterococcus faecalis* does not induce resistance to MFA poisoning since all inoculated animals developed clinical signs of poisoning that evolved to death, as did the sheep that did not receive the bacterium.

In 2012, Davis and collaborators (Davis et al. 2012) isolated an anaerobic bacterium belonging to the phylum *Synergistetes* (strain MFA1). They detected that this bacterium was capable of degrading fluoroacetate, suggesting its use in the control of poisoning by this compound and by plants that present it as a toxic principle. In the same year, aerobic bacteria were isolated from the soil and plants containing MFA: *Paenibacillus* sp. (ECPB01), *Burkholderia* sp. (ECPB02), *Cupriavidus* sp. (ECPB03), *Staphylococcus* sp. (ECPB04), *Acylobacter* sp. (ECPB05), *Ralstonia* sp. (ECPB06), and *Stenotrophomonas* sp. (ECPB07). They were also isolated from goat rumen as bacteria *Pigmentiphaga kullae* (ECPB08) and *Acylobacter dichloromethanicus* (ECPB09). All these bacteria were able to degrade MFA, suggesting their use in the protection against poisoning by MFA, and by plants containing MFA as a toxic principle (Camboim et al. 2012a, 2012b).

P. kullae and *A. dichloromethanicus* isolated from goat rumen were orally administered in combination to goats for ten days. After administration of the bacteria, 5g/kg of *A. septentrionalis* was administered aiming to evaluate the efficiency of these bacteria in the protection against plant poisoning. The researchers found that the animals that received the bacteria were resistant to higher doses of the plant, and took longer to present clinical signs of poisoning compared with the goats that did not receive the bacteria, demonstrating their ability to induce resistance to poisoning by *A. septentrionalis* (Pessoa et al. 2015).

Silva et al. (2016) also used bacteria isolated from soil and plants containing MFA. *Paenibacillus* sp. and *Cupriavidus* sp. were administered in combination to a group of goats (Group 1), and *Burkholderia* sp. and *Ralstonia* sp. were also administered in combination to another group of goats (Group 2). A third group of goats served as controls and received no bacteria (Group 3). Bacterial administration was carried out daily for ten days, and as of the 10th day, 5g/kg *A. septentrionalis* were daily administered simultaneously with the bacteria. The animals in Group 2 ingested *A. septentrionalis* for 30 days and did not develop signs of poisoning, proving the induction of poisoning resistance by *Burkholderia* sp. and *Ralstonia* sp. *Paenibacillus* sp. and *Cupriavidus* sp. induced partial resistance, since all goats showed clinical signs of poisoning, but they were milder and started later compared with those in Group 3. All goats in Group 3 showed severe clinical signs of poisoning as of the third day of plant administration, and in two of these animals the signs evolved to death. It was also found that the goats in Group 2 were able to ingest larger amounts of the plant without developing clinical signs of poisoning compared with those in Group 3. Silva et al. concluded that bacteria should be administered continuously, without interruption, and suggested using these bacteria as added probiotics in animal feed in areas where MFA-containing plants are endemic.

After experimental resistance induction through inoculation of *Paenibacillus* sp., *Cupriavidus* sp., *Burkholderia* sp. and *Ralstonia* sp., researchers tested the ability of goats to maintain this resistance to naturally eating *A. septentrionalis* under field conditions. The study was conducted in Pernambuco from 2014 to 2015. The goats, which were resistant to experimental poisoning by administration of bacteria previously mentioned (Silva et al. 2016), were released to graze in an area infested by the plant. All animals ingested the plant daily, showed clinical signs of poisoning and died; however, it took up to 55 days for the animals that received the bacteria to develop clinical signs of poisoning and die, whereas animals that did not receive the bacteria developed clinical signs and died within 27 days of plant ingestion (Pessoa et al. 2018). Another bacterium recently used to control plant poisoning containing MFA, *Herbaspirillum seropedicae*, is commonly used in agriculture to improve the growth of crops such as corn and soybeans, and has a gene capable of encoding the production of the dehalogenase enzyme, which makes it able to degrade MFA (Pedrosa et al. 2011). This bacterium was administered orally daily to six goats (Group 1), and as of the 10th day of administration, they simultaneously received the bacterium and the plant, *A. septentrionalis*, until clinical signs of poisoning were observed. Other six goats served as controls and received only the plant (Group 2). It took the goats in Group 1 16.16 ± 2.56 days to develop clinical signs of poisoning, and they were able to ingest 80.83 ± 12.81 g/kg of the whole plant. Those in Group 2 presented signs after 7.83 ± 3.81 days, and ingested 39.16 ± 19.08 g/kg of the whole plant. Because *H. seropedicae* is an endophytic bacterium that is capable of colonizing plants, it was cultivated and later used to spray some plants of the species *A. septentrionalis*, aiming to evaluate whether there was a reduction in the amount of MFA present in them after spraying. Before spraying, the concentration of MFA found in the plants was 1.21 ± 0.53 μ g/mg, and a significant reduction in the amount of MFA (0.24 ± 0.05 μ g/mg) was observed eight days after spraying, suggesting the use of endophytic bacteria, which hydrolyze MFA, as a way to reduce the content of this compound in plants (Pessoa et al. 2019). Further studies should be conducted to prove these results and demonstrate the permanence of *H. seropedicae* in *A. septentrionalis* plants or in other species containing MFA.

The use of MFA-degrading microorganisms, either through their administration to animals or their direct use on plants containing MFA, is an efficient alternative to control poisoning by plants containing this compound in ruminants.

Use of conditioned food aversion to prevent ingestion of MFA-containing plants

The conditioned food aversion technique is commonly used in ruminants to induce non-consumption of toxic plants. It consists of administration of a substance, most often lithium chloride (LiCl), via gastric tube or rumen fistula soon after ingestion of the toxic plant. Lithium chloride is the most widely used emetic in both animals and humans, and causes nausea with no severe side effects. The animal associates the disorder caused by LiCl to the taste of the food and, consequently, will avoid this taste, thus avoiding ingestion of the plant (Provenza et al. 1994, Ralphs et al. 2001). However, an important factor that hinders induction of aversion to the ingestion of a specific plant is social facilitation, that is,

if the animals that were submitted to aversive conditioning watch other animals eating the plant, they may ingest it again (Ralphs & Provenza 1999). Because some MFA-containing plants are highly palatable to ruminants, such as those of genus *Palicourea*, this technique has been used in some experiments with the purpose of promoting the control of poisoning by plants containing this compound.

Barbosa et al. (2008) performed the conditioned food aversion test with *Mascagnia rigida* ingestion (currently classified as *Amorimia septentrionalis*). To this end, goats unfamiliar with the plant received 100mg/kg LiCl orally daily 15 min after ingestion of *A. septentrionalis* leaves until they stopped eating it. The control group received only water, also orally. On days 10, 17 and 24 after induction of aversion, the plant was again offered to the goats to evaluate whether aversion persisted. On the first test day, no difference was observed between the groups; however, on day 2, five goats that had received LiCl did not ingest the plant, and as of day 3 after aversion induction, all goats treated with LiCl did not ingest the plant. The goats who did not receive LiCl ingested increasing amounts of the plant throughout the experiment, indicating that LiCl induced aversion to plant ingestion, at least temporarily.

Silva & Soto-Blanco (2010) conducted the conditioned food aversion test with ingestion of *Mascagnia rigida* (currently classified as *A. septentrionalis*) in sheep that had never had contact with it. To this end, the sheep were divided into 2 groups: Lithium Group and Control Group. In the lithium group, the sheep received 150mg/kg LiCl orally 15 min after the plant leaves were offered. Unlike the animals in the lithium group, those in the control group received water, following the same methodology. Silva et al. observed that the sheep that received LiCl no longer ingested the plant as of day 3, and aversion persisted for up to 70 days after induction.

Conditioned food aversion to *P. aeneofusca* was tested in goats. Oliveira and collaborators divided the animals into two groups (Treated Group and Control Group). In the Treated Group, the animals received 175mg/kg LiCl via rumen tube 10 minutes after plant ingestion, whereas the goats in the Control Group received only water, following the same methodology. The aforementioned authors concluded that aversion to *P. aeneofusca* began five days after treatment commencement and persisted for up to 90 days (Oliveira et al. 2014).

Brito et al. (2016) tested the technique of conditioned food aversion to *P. aeneofusca* in cattle. To this end, animals were divided into two groups of six: one group was composed of animals treated with LiCl at a dose of 175mg/kg shortly after consumption of *P. aeneofusca* leaves and the other group comprised animals that received 1mL/kg of water, following the same methodology used for the other group. After induction, the animals in both groups were released to graze in separate paddocks, aiming to avoid social facilitation, infested by the plant once every 30 days, where they remained for a maximum of 15 consecutive days, or until they developed clinical signs of poisoning. After this period, the animals were taken to graze in areas free of the plant; this procedure was repeated for 12 months, always alternating the grazing of the animals between areas with presence of the plant and free of it. The cattle that received LiCl did not ingest the plant in the field at any time, and aversion persisted throughout the experiment. In contrast, the control animals ingested the plant

continuously, and it was necessary to remove them from the pasture. Brito et al. conclude that conditioned LiCl aversion in cattle is effective to prevent *P. aeneofusca* consumption for at least 12 months.

These studies have shown that conditioned food aversion is an efficient technique to prevent ingestion of some MFA-containing plants; however, it is difficult to be applied in commercial farms, especially in those with large number of animals.

Use of acetamide in the treatment and control of poisoning by MFA-containing plants

The use of some compounds to treat poisoning by MFA-containing plants in ruminants has been reported in several studies. These compounds can change how MFA acts in the animal organism and, consequently, reduce its toxicity.

Acetamide is amide derived from acetic acid, and is represented by the molecular formula CH_3CONH_2 . This compound can be used to prevent poisoning by plants that contain MFA in ruminants, as it has a protective effect against the action of MFA, i.e., it acts as an "acetate donor", preventing the "lethal synthesis" of MFA in the organism. However, its effectiveness depends on several factors, such as the dose used, the interval between the onset of clinical signs and administration of the acetamide dose, and the toxicity of the plant that is causing poisoning (Egyed & Schultz 1986, Peixoto et al. 2011b).

Peixoto et al. (2011a) administered 0.5mg/kg MFA to cattle, and soon after divided the animals into two groups: one group received 0.38g/kg acetamide and the other 2g/kg acetamide. Other cattle were offered 1g/kg of *Palicourea marcgravii* leaves, followed by 1g/kg acetamide. Acetamide was able to protect the animals from development of clinical signs of poisoning at almost all doses used. Only the dose of 0.38g/kg acetamide was not efficient, and one bovine presented clinical signs that evolved to death. However, when the experiment was repeated eight days later, without acetamide, all cattle developed clinical signs and died, which proves the protective effect of acetamide, but also reinforces that protection is not long-lasting.

The use of this compound as an antidote to poisoning by MFA-containing plants in ruminants is limited, because for treatment to be effective it must be used soon after or a few hours before ingestion of the plant, which becomes unfeasible since the evolution of poisoning is superacute; in addition, the availability and cost of acetamide limit its use (Peixoto et al. 2011a).

CONCLUSIONS

In conclusion, several techniques have been experimentally effective in controlling poisoning by MFA-containing plants, but none of them can be used without restriction commercially.

One of the most efficient measures was the use of MFA-degrading bacteria, but these bacteria must be continuously administered. Therefore, it is necessary to develop commercial products containing strains that can be administered to animals continuously, probably as probiotics.

It also seems promising to infect MFA-containing plants with bacteria that degrade it, thereby reducing their toxicity. Further studies to improve the existing techniques are needed, in order to make them accessible to producers.

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Biochemical, endocrine, and histopathological profile of liver and kidneys of sheep with pregnancy toxemia¹

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ABSTRACT. Souto R.C.J., Afonso J.A.B., Mendonça C.L., Dantas A.F.M., Cajueiro J.F.P., Gonçalves D.N.A., Oliveira Filho E.F. & Soares P.C. 2019. **Biochemical, endocrine, and histopathological profile of liver and kidneys of sheep with pregnancy toxemia.** *Pesquisa Veterinária Brasileira* 39(10):780-788. Clínica de Bovinos, Universidade Federal Rural de Pernambuco, Campus Garanhuns, Av. Bom Pastor s/n, Cx. Postal 152, Boa Vista, Garanhuns, PE 55292-270, Brazil. E-mail: rodolfojcsouto@gmail.com

The objective of the present study was to characterize the biochemical, hormonal, and mineral profile, and histopathology of the liver and kidneys, related to the severity of natural cases of pregnancy toxemia (PT) in sheep. A total of 45 sheep with PT were analyzed at the "Clínica de Bovinos", Campus Garanhuns-UFRPE. The animals were submitted to clinical examination, followed by the collection of blood and urine. A necropsy was performed on thirteen animals that died and a histopathological examination was performed on samples of liver and kidneys. Increased creatinine, urea, glucose, fructosamine, non-esterified fatty acids, β -hydroxybutyrate, cortisol, chlorine, amylase, aspartate aminotransferase, gamma-glutamyltransferase, and folic acid were found, whereas insulin, potassium, and total and ionizable calcium presented low values for the species. Total protein, albumin, globulin, sodium, magnesium, and vitamin B12 remained within the normal range. In the macroscopic analysis of the liver, it was possible to observe an increase in organ and border size, yellowish coloration and parenchyma that varied from firm to friable. Vacuolation of the hepatocytes and renal tubular cells was observed. Metabolic disorders in sheep associated with hepatic and renal lesions are more apparent in overweight animals. With the greater impairment in hepatic and renal function, the clinical prognosis of animals with a high body score should be considered reserved.

INDEX TERMS: Biochemistry, endocrine, histopathology, liver, kidneys, sheep, pregnancy toxemia, clinical biochemistry, metabolic profile, energy deficiency, pregnancy toxemia, anatomopathological lesions, ovine.

RESUMO. [Perfil bioquímico, endócrino e histopatológico do fígado e rins de ovelhas com toxemia na prenhez.] Objetivou-se caracterizar o perfil bioquímico, hormonal,

mineral e a histopatologia hepática e renal, relacionando com a severidade de casos naturais de toxemia da prenhez (TP) em ovelhas. Analisou-se 45 ovelhas na Clínica de Bovinos, Campus Garanhuns-UFRPE, com TP. Foram submetidas ao exame clínico, seguido de coleta de sangue e urina. Treze animais que vieram a óbito foram necropsiados, nas amostras de fígado e rins realizou-se exame histopatológico. Revelou-se elevação de creatinina, ureia, glicose, frutossamina, ácidos graxos não esterificados, β -hidroxibutirato, cortisol, cloro, amilase, aspartato aminotransferase, gama glutamiltransferase e ácido fólico, enquanto insulina, potássio, cálcio total e ionizável apresentaram valores abaixo para os da espécie. A proteína total, albumina, globulina, sódio, magnésio e vitamina B12 mantiveram-se dentro da normalidade. Na análise

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macroscópica do fígado, pôde-se observar aumento de tamanho do órgão e dos bordos, coloração amarelada e parênquima que variava de firme a friável. Observou-se vacuolização dos hepatócitos e das células tubulares renais. Os transtornos metabólicos nas ovelhas associados às lesões hepáticas e renais são mais aparentes nos animais com sobrepeso. Com o maior comprometimento da função hepática e renal, o prognóstico clínico dos animais com escore corporal elevado há de ser considerado reservado.

TERMOS DE INDEXAÇÃO: Endócrino, histopatologia, fígado, rins, ovelhas, gravidez, bioquímica clínica, perfil metabólico, deficiência de energia, toxemia da prenhez, lesões anatomopatológicas, ovinos.

INTRODUCTION

Pregnancy toxemia (PT) is a metabolic disorder that occurs in sheep and goats carrying twins or triplets, although it may occur in simple pregnancies with large fetuses, during the last 4 to 6 weeks of gestation, when there is an increase in energy demand due to rapid fetal growth. This condition, associated with factors that induce a reduction in food intake, such as obesity and inadequate diet supply, contribute to the duration and magnitude of the negative energy balance and predisposition to the onset of the disease in its subclinical or clinical form (González & Silva 2006, Radostits et al. 2007).

The disease manifests more frequently in intensive than extensive systems of production (Corrêa et al. 2010), and can be classified as type I when linked to undernourishment during pregnancy or type II which usually occurs in sheep that are very obese (Ortolani 2004). The clinical manifestation is characterized by depression, weakening, reduction in food intake, respiratory alterations, and nervous symptoms. There is hypoglycemia, hyperketonemia, ketonuria, and fat deposition in the liver, which can be severe (Hefnawy et al. 2011, González et al. 2012, Lima et al. 2012).

A framework of metabolic acidosis has been observed in PT, with an increase in the concentration of NEFAs, β -hydroxybutyrate, and anion gap, and a decrease in blood pH, bicarbonate concentration, and base excess, as well as marked alterations in the electrolytic profile involving the calcium, phosphorus, chlorine, sodium, and potassium ions (Hefnawy et al. 2011, González et al. 2012, Lima et al. 2012). Furthermore, hormonal alterations are closely involved in the development of ketosis in ruminants (Brockman 1979).

Hepatic and renal function are usually compromised as a result of fatty infiltration, which due to its severity may interfere with gluconeogenic capacity, contributing to ketosis and azotemia indicative of severe renal damage (Parry & Taylor 1956, Wastney et al. 1983, Van Saun 2000, Cal et al. 2009, Yarim & Ciftci 2009). PT in sheep is a complex disease and often presents poor treatment prognosis. Thus, a better understanding is required in terms of its biochemical and hormonal behavior and the consequences of these alterations on vital organs such as the kidneys and liver, especially in the occurrence of spontaneous cases.

Studies that report the dynamics of biomarkers indicating the severity of cases of pregnancy toxemia are still scarce, particularly in relation to treatment of the situation. In view of the above, the objective was to characterize the profile of different biochemical, hormonal, and histopathological characteristics of the liver and kidneys of natural cases of PT in sheep.

MATERIALS AND METHODS

Data from 45 sheep reared in the state of Pernambuco were included, of the breeds Santa Inês, Dorper, and mixed, aged from one to seven years, with a mean weight of 67kg, mostly multiparous, and predominantly submitted to semi-intensive and intensive rearing. The majority of animals received a body score of three or above and their diet was based on concentrates. The clinical examination was performed according to Radostits et al. (2007), and the animals were submitted to an ultrasound examination to determine fetal viability.

Blood samples were collected through jugular venipuncture using a 25x8mm needle into vacutainer siliconized tubes and centrifuged⁶ at 3500RPM for five minutes with anticoagulant sodium fluoride/oxalate to obtain plasma and determination of glucose. The samples obtained in tubes without anticoagulant, to obtain the serum, were used in the biochemical and hormonal analyzes. The samples were conditioned in the form of aliquots of serum and plasma in Eppendorf tubes and stored in an ultra-freezer at a temperature of -80°C⁷.

The evaluated components were: total protein⁸, albumin⁸, urea⁸, creatinin⁸, fructosamine⁸, amylase CNPG⁸, aspartate aminotransferase (AST)⁸, gamma-glutamyltransferase (GGT)⁸, creatine kinase (CK)⁸, calcium liquiform (total calcium)⁸, phosphorus UV Liquiform⁸, magnesium⁸, and chlorides⁸ performed in a semi-automated biochemical analyzer, LabQuest⁸ using commercial reagents⁸. The variables sodium⁹ and potassium were determined⁹ using a flame photometer of the brand Benfer⁹. The globulin values were obtained by subtracting the albumin value from the total protein, the ionized calcium using a calculation involving the value of albumin, total protein, and total calcium (methodology used by the manufacturer - Doles¹⁰), and the calcium:phosphorus ratio by dividing the value of total calcium by the value of phosphorus. The non-esterified fatty acids (NEFA) and β -hydroxybutyrate (BHB) were determined according to commercial reagents (Randox Laboratories Ltd)¹¹ and for the hormonal determinations of cortisol and insulin, folic acid, and vitamin B12 the electrochemiluminescence technique was used¹², with commercial reagents (Cobas[®])¹². Evaluation of ketone bodies was carried out on urine collected by means of spontaneous micturition using urinalysis reagent strips (Dirksen et al. 1993).

The sick animals that had died were submitted to necropsy. The kidneys and liver were collected for macroscopic and histological analysis. The collected material was fixed in 10% formalin, embedded in paraffin, cut 4-5 micrometers thick, and stained with hematoxylin-eosin (HE). The slides were read using light microscopy. In order to evaluate the degree of the lesions as a function of the body score, two groups were established: Group 1 with a score of up to three (n=7) or Group 2 with a score higher

⁶ Centrifuga Fanem Ltda Baby I, Mod. 206, Av. General Ataliba Leonel 1790, São Paulo, SP 02033-020, Brazil.

⁷ Ultralow freezer NuAire Inc., 2100 Fernbrook Lane N. Plymouth, MN 55447, USA.

⁸ Labtest Diagnóstica S.A., Av. Paulo Ferreira da Costa 600, Lagoa Santa, Minas Gerais, MG 33400-000, Brazil.

⁹ Benfer, Produtos para laboratórios, Rua Padre Agostinho Poncet 74, São Paulo, SP 02408-040, Brazil.

¹⁰ Doles, Rodovia BR-153 Km 493, Lote 7, Chácara Retiro, Goiânia, GO 74665-833, Brazil.

¹¹ Randox Laboratories Ltd, Ardmore, Diamond Road, Crumlin, Co. Antrim, United Kingdom BT 29 4QY, UK.

¹² Cobas e 411, Roche Sistemas de Diagnósticos Ltda, Avenida Eng. Billings 1729, Jaguaré, São Paulo, SP 05321-900, Brazil.

than three ($n=6$). The histopathological lesions of the liver were classified by the degree of intensity and distribution in the three areas of the parenchyma, area 1 comprising the periportal, area 2 the midzonal, and area 3 the centrilobular.

The data were processed considering measures of central tendency and absolute and relative frequencies. Pearson's correlation coefficient was determined by association between pairs of variables. The significance obtained in the correlation was performed according to Little & Hills (1978). The data were analyzed using the Statistical Analysis System (SAS 2009). In relation to the liver and kidneys the data were recorded descriptively.

The project obtained a positive opinion from the Ethics Committee on the Use of Animals (CEUA), "Universidade Federal Rural de Pernambuco" (UFRPE), license number: 024/2012 of CEPE-FRPE according to COBEA standards and the National Institute of Health Guide for Care and Use of Laboratory Animals.

RESULTS

Of the 45 sheep with PT evaluated, 82.90% were normoglycemic and/or hyperglycemic, while only 17.10% were hypoglycemic. The mean number of lambs per birth per ewe was higher

in those that were hyperglycemic and hypoglycemic, while normoglycemic sheep presented the lowest mean. The lowest number of deaths occurred in sheep in a hypoglycemic phase 1/7 (14.28%), followed by hyperglycemic 4/23 (17.39%), and normoglycemic 4/11 (36.36%) (Table 1). In the sheep from which urine was harvested, ketonuria was found in all of them.

High values of creatinine, urea, fructosamine, NEFes, BHB, cortisol, chlorides, amylase, AST, GGT, and folic acid were also found, whereas the variables: insulin, potassium, and total and ionizable calcium were below reference values for the species. Other biochemical and electrolytic parameters, such as total protein, albumin, globulin, vitamin B12, phosphorus, sodium, and magnesium remained within the normal range (Table 2-6).

A high-intensity negative relationship was observed between the serum cortisol concentration and insulin ($r = -0.71$), a high intensity positive relationship between creatinine and urea ($r = 0.71$), and between total calcium and insulin ($r = 0.65$), and a positive moderate ratio was observed between glucose and insulin concentration ($r = 0.47$) (Fig.1).

Table 1. Mean and standard deviation values ($\bar{x} \pm s$) of the glycemic profile, number of animals, number of offspring, and clinical outcome of sheep with PT, attended at the "Clínica de Bovinos", Campus Garanhuns-UFRPE

Clinical condition	Glycemic condition (mmol/L)	Number of animals	Number and average of lambs/ewes	Outcome	
				Healing	Death
Hypoglycemics	2.43 $\pm$ 0.29	07 (17.10%)	14 ($\bar{x}=2.00$)	6	1
Normoglycemics	3.83 $\pm$ 0.40	13 (31.70%)	22 ($\bar{x}=1.83$)	7	4
Hyperglycemics	7.34 $\pm$ 1.72	21 (51.20%)	44 ($\bar{x}=2.09$)	19	4

Table 2. Mean, standard deviation, minimum and maximum values of biochemical (protein) parameters in sheep affected with PT, attended at the "Clínica de Bovinos", Campus Garanhuns-UFRPE, from 2007 to 2012

Parameters	Means	Standard deviation	Minimum	Maximum	Reference values
Creatinine (μ mol/L)	175.82	161.77	43.32	685.10	106.08 - 167.96 ^a
Urea (mmol/L)	10.25	8.86	2.85	48.84	2.91 - 7.28 ^a
Total Protein(g/L)	75.20	15.08	45.40	108.20	60.0 - 79.0 ^a
Albumin (g/L)	29.35	8.79	15.30	64.90	24.0 - 30.0 ^a
Globulin (g/L)	45.82	15.07	7.20	83.50	35 - 57 ^a

^a Source: Kaneko et al. (2008).

Table 3. Mean, standard deviation, minimum and maximum values of biochemical (energy) parameters, in sheep affected with PT, attended at the "Clínica de Bovinos", Campus Garanhuns-UFRPE, from 2007 to 2012

Parameters	Means	Standard deviation	Minimum	Maximum	Reference values
Glucose (mmol/L)	5.39	2.42	2.22	11.22	2.8-4.48 ^a
Fructosamine (μ mol/L)	195.33	44.61	119.36	337.51	172 $\pm$ 2.0 ^b
NEFA (mmol/L)	1.15	0.59	0.23	3.10	< 0.4 ^a
BHB (mmol/L)	1.39	1.18	0.30	5.08	< 0.6 ^c
Folic Acid (ng/ml)	11.63	7.38	2.76	20.00	1.94-3.16 ^d
Vitamin B ₁₂ (pg/ml)	1781.88	459.65	279.00	2000.00	1494.71-1842.89 ^d

Source: ^a Kaneko et al. (2008), ^b Cantley et al. (1991), ^c Contreras & Wittwer (2000), ^d Carvalho (2013).

Table 4. Mean, standard deviation, minimum and maximum values of biochemical (enzyme) parameters, in sheep affected with PT, attended at the “Clínica de Bovinos”, Campus Garanhuns-UFRPE, from 2007 to 2012

Parameters	Means	Standard deviation	Minimum	Maximum	Reference values
Amylase (U/L)	33.60	26.79	7.90	134.40	7.76-11.5 ^a
AST (U/L)	308.21	133.53	107.36	639.05	60-280 ^b
GGT (U/L)	83.81	52.47	38.25	237.15	20-52 ^b

Source: ^a Araújo & Silva (2008), ^b Kaneko et al. (2008).**Table 5. Mean, standard deviation, minimum and maximum values of biochemical (electrolyte) parameters, in sheep affected with PT, attended at the “Clínica de Bovinos”, Campus Garanhuns-UFRPE, from 2007 to 2012**

Parameters	Means	Standard deviation	Minimum	Maximum	Reference values
Total Calcium (mmol/L)	2.1	0.74	0.65	18.70	2.88-3.2 ^a
Ionized Calcium (mmol/L)	1.15	0.38	0.36	2.05	1.49-1.73 ^a
Ca:P (mmol/L)	1.22	0.71	0.21	3.55	1:4-1:6 ^c
P (mmol/L)	2.06	0.94	0.52	5.23	1.0-2.0 ^b
Mg (mmol/L)	1.00	0.27	0.48	1.80	0.7-1.1 ^b
Na (mmol/L)	139.25	9.60	120.00	161.00	139-152 ^a
K (mmol/L)	3.77	0.72	2.40	5.50	4.4-7.2 ^b
Cl (mmol/L)	110.78	8.10	86.70	122.70	95-103 ^a

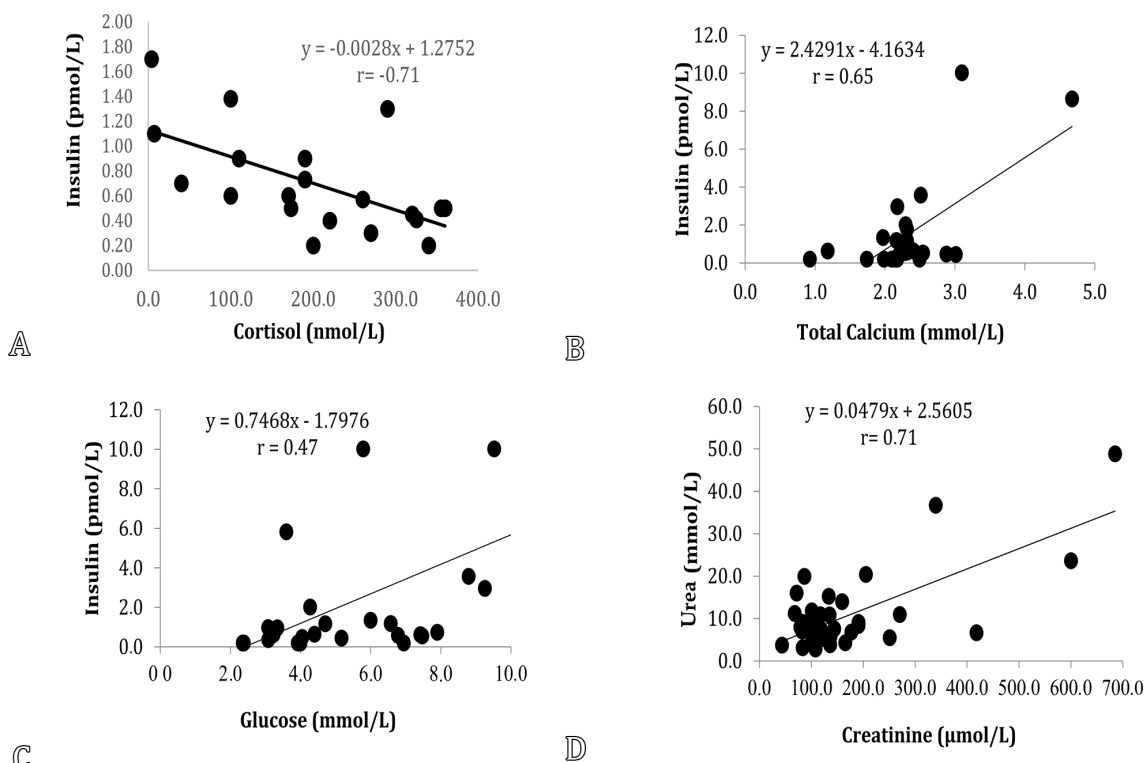
Source: ^a Kaneko et al. (2008), ^b Contreras & Wittwer (2000), ^c Barioni et al. (2001).

Fig.1. Relationship between metabolic profile components in ewes with pregnancy toxemia.

Table 6. Mean, standard deviation, minimum and maximum values of hormonal parameters, in sheep affected with PT, attended at the “Clínica de Bovinos”, Campus Garanhuns-UFRPE, from 2007 to 2012

Parameters	Means	Standard deviation	Minimum	Maximum	Reference values
Cortisol (nmol/L)	287.76	348.53	3.48	1345.00	62±10 ^a
Insulin (pmol/L)	2.33	3.53	0.20	14.27	117±0.6 ^b

Source: ^a Kaneko et al. (2008), ^b Henze et al. 1998.

The majority of livers of the animals with Group 1 PT (n=7) presented a slight increase in liver and border size. The yellowish coloration on the surface was also present at the cut. In the animals of Group 2 (n=6) these findings were more expressive regarding the intensity (Fig.2). The kidneys did not present marked alterations, with a pale surface appearance in some of the lean animals, and slight yellowish coloration in fat animals. Of the lesions found in the liver and kidneys, only cell vacuolization was considered to be directly related to the metabolic disorder. Thus, we attempted to establish a parallel between ECC (Groups 1 and 2), the degree of intensity, and distribution of cellular vacuolations.

Regarding the findings for the liver, in Group 1 42.86% (3) presented unusual vacuolization while in 57.14% (4) of the cases it was discrete. The lesions were distributed as follows: in 42.86% (3), these were located in zone I, 28.57% of the animals (2) presented vacuolization in zones 1 and 2, and 28.57% (2) were in zone 3. Regarding the renal lesions, only

28.57% of the animals (2) presented unusual vacuolations in tubular epithelial cells.

With respect to the degree of vacuolization in the liver of the animals in Group 2, severe lesions were found in 33.33% (2), moderate lesions in 33.33% (2), discrete lesions in 16.67% (1), and unusual lesions in only 16.67% (1). The hepatic lesions were distributed as follows: 66.67% (4), presented lesions in the three zones, 16.67% (1) in zones 1 and 3, and 16.67% (1) in zone 3 (Fig.3A). Regarding the renal lesions: 33.33% (2) presented a marked lesion of vacuolations in tubular epithelial cells, in 33.33% (2) these were sparse, but in one of these the tubular cells affected were in the cortical and cortical-medullary regions (Fig.3B). The others did not present fatty infiltration in their structure.

DISCUSSION

The increase in blood glucose in the majority of animals is in agreement with that described in the literature when defining PT (Scott et al. 1995, Corrêa et al. 2010), which reports a decrease in serum glucose concentration as the most frequent finding, which, according to Afonso (2006) is initially found in the disease, however with disease progression, glycemic levels tend to stabilize, with some sheep presenting a framework of hyperglycemia, and in these cases the prognosis is unfavorable as the fetuses are probably dead. According to Pereira et al. (2010), at the end of gestation sheep present a lower ability to metabolize glucose, with rising glycemic levels possibly due to increased insulin resistance in peripheral tissues. However, this condition was not the only one found among the affected animals, normoglycemic sheep and a minority of hypoglycemic sheep composed the group, which is in agreement with the findings of Henze et al. (1998).

These findings focus attention on the importance of analysis of the glycemic condition of sheep with PT prior to the therapeutic administration of glucose by the parenteral route, considering that its administration when not necessary could lead to hyperglycemic shock and death of the female. The higher survival rate observed among hypoglycemic ewes may be related to the lower degree of stress and better



Fig.2. Enlarged liver with yellowish coloration in sheep with pregnancy toxemia.

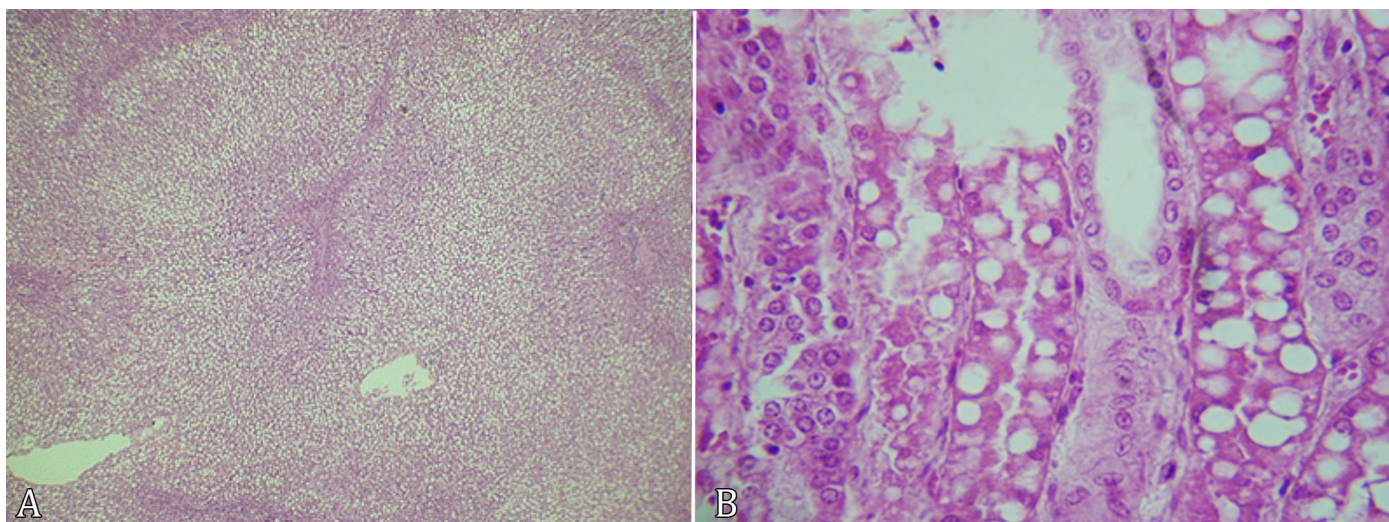


Fig.3. (A) Massive vacuolization of the hepatocyte cytoplasm in the three zones of the hepatic lobe in sheep with pregnancy toxemia. HE, obj.40x. (B) Vacuolization in tubular epithelial cells in sheep with pregnancy toxemia. HE, obj.40x.

response to hypoglycemia compared to other glycemic conditions, provided that it is not severe enough to cause irreversible damage to the nervous system (Jeffrey & Higgins 1992), findings in accordance with the results of Lima et al. (2012) who observed better results regarding fetal viability when intervention in goats with PT was performed in a hypoglycemic phase.

The albumin indices, which were found unchanged, resemble the reports by Cantley et al. (1991), Andrews et al. (1996), and VanSaun (2000) in sheep with metabolic disorder, however values lower than those considered normal, were verified by Yarim & Ciftci (2009) and Hefnawy et al. (2011) which justify the reduction through hepatic or renal failure, as its synthesis occurs in the liver, due to the presence of fatty infiltration in animals with high lipomobilization. In relation to the total protein and globulin, for which indices were maintained within the normal range for the species, this differs from the findings of Cerón et al. (1994) and Yarim & Ciftci (2009), where a reduction in these variables was verified in goats and sheep with PT and the authors explained this alteration through impaired hepatic function. Elevation in the values found for total serum protein in other studies is justified by the different degrees of dehydration intensity that animals with the disease can present (Cantley et al. 1991, VanSaun 2000).

The azotemia framework occurred due to possible renal alteration, considering that according to Reece (2006), free creatinine in the blood is not reused and is excreted in the urine. Reductions in creatinine clearance values are associated with elevated plasma concentrations and represent a loss in nephron numbers or reduced renal function. According to Afonso (2006) and Santos et al. (2011) a reserved prognosis should be attributed to the appearance of renal injury when evidenced by elevated creatinine concentration in cases of PT. Urea elevation may also be related to renal alteration, according to González & Silva (2006) the kidneys have a high ability to excrete it, being filtered in the glomerulus and partially resorbed passively in the tubules. In ruminants, it can be excreted gastrointestinally, so that normal or not much elevated values can be found in cases of renal failure. According to Wastney et al. (1983), high concentrations associated with creatinine are indicative of severe renal failure in sheep with PT. However, the main limitation of these two variables is that they detect renal failure when 75% of the affected nephrons are reached (González & Silva 2006).

Regarding the high values of fructosamine, this is due to the state of hyperglycemia found in the majority of affected sheep, a result that was observed by Santos et al. (2011), and distinct from that found by Cantley et al. (1991) who reported hypoglycemia in sheep with PT. It is important to study this variable as it is a stable ketoamine formed when glucose reacts non-enzymatically with amino groups in proteins, its concentration controlled by the equilibrium between the rates of synthesis and removal. Thus, the serum fructosamine concentration will increase with prolonged hyperglycemia or prolonged hyperproteinemia, tending to fall with increased protein renewal or prolonged hypoglycemia (Bernstein, 1987). Therefore, the concentration of fructosamine in peripheral blood varies according to the long-term state of glycemia and/or the protein metabolism of an individual, in the previous two week period (Murphy et al. 1997). Its use in cases of PT is even more important since according to Armbruster (1987) it is not subject to changes due to transient hyperglycemia, and that a single measurement of glucose shows only the momentary concentration which is subject to rapid and

frequent changes, dependent on daily variations, dietary factors, and individual factors.

The elevation in NEFAs found in sheep with PT, according to Andrews (1997), portrays the magnitude of the metabolic disorder, and is explained by the lipolysis that occurred, indicating the existence of fat mobilization to meet greater energy demand from the sheep and fetuses. Increases in NEFAs were also found in sheep with PT by Sigurdsson (1991), VanSaun (2000), and Santos et al. (2011). However, for Regnault et al. (2004) and Corrêa et al. (2010), this elevation may point to a deleterious effect of NEFAs on the sensitivity and action of insulin, as well as knowing that these act by inhibiting the secretion of this hormone by β -pancreatic cells, and may even stimulate cellular apoptosis pathways. In addition, lower uptake of glucose, secondary to defects in the transduction of the insulin signal, has been reported, thus the glucose is spared and made available for use in the placenta and fetuses.

The elevation in β -hydroxybutyrate occurred due to the high degree of lipomobilization found with the consequent synthesis of NEFAs, which undergo β -oxidation and in excess form large amounts of ketone bodies. An increase in β -hydroxybutyrate concentration was observed by Scott et al. (1995), Andrews et al. (1996), Andrews (1997), Moghaddam & Hassanpour (2008), Bani Ismail et al. (2008), and Hefnawy et al. (2010) in sheep and goats affected by the disease and although the ketone bodies are produced by the liver, they are used by other tissues, and ketosis could be the result of their underuse or excessive production. According to Harmeyer & Schlumbohm (2006), this increase is caused by a reduction in the ability of pregnant females to use β -hydroxybutyrate and induces several adverse effects on energy balance and glucose metabolism, facilitating the development of PT, especially in sheep with twin/multiple pregnancies.

With respect to folic acid, information regarding its relationship with the disease under study is scarce, but the result found is similar to that of Carvalho (2013), who found an increase in the serum concentration of folic acid in sheep with PT in relation to healthy animals receiving diets with low and high energy density. Zinn et al. (1987) and Chiquette et al. (1993) with folic acid supplementation in bovines, observed a significant increase in propionate concentration and a tendency to reduce ruminal pH. In this way it would be interesting to understand the dynamics of this vitamin related to the metabolic disorders that occur in sheep.

The high levels found for vitamin B12 were similar to those reported by Carvalho (2013), both in sheep receiving diets with high energy density and in diseased sheep with PT, this fact is justified, since the majority of the affected sheep were reared in semi-intensive and intensive systems, with diets rich in energy concentrate, which reflect in a body score above three in most animals. Therefore, due to the scarcity of information available, the determination of serum levels of vitamin B12 in sheep with PT is of extreme interest, since ruminants need, at the cellular level, all the vitamins for the proper functioning of their body and cobalamin (vitamin B12) participates in several metabolic activities.

According to Strobel (1992), vitamin B12 is fundamental in the energetic metabolism of sheep, since it acts as a cofactor in reactions that promote the production of propionic acid, the main precursor of glucose synthesized in the liver of sheep. Therefore, if a ruminant is deficient in vitamin B12 it presents decreased energy efficiency (Contreras & Wittwer 2000).

The elevation in amylase levels in sheep with PT may be related to an excessive supply of carbohydrates, since, according to Van Hellen et al. (1978), Harmon (1993), and Kaneko et al. (2008), the amylase synthesis in the pancreas in animals with high carbohydrate diets is increased, as it is responsible for catalyzing the specific hydrolysis of the α -1,4-glycosidic bonds that are present in the starch, which escape fermentation and are digested until glucose in the small intestine. On the other hand, the increase in amylase may be a result of renal damage, corroborating with the elevated levels of serum urea and creatinine found in this regard. González & Silva (2006), affirm that hyperamylasemia can be caused by decreased glomerular filtration, since it is eliminated in the urine.

The increase in liver enzymes AST and GGT was due to hepatic damage, since AST, according to González & Silva (2006), is a good indicator of hepatic functioning in ruminants. In cases of PT, an increase in AST was observed by Henze et al. (1998) and Barakat et al. (2007). According to Kabakci et al. (2003) concentrations of the enzyme remained unchanged in sheep even 20 days after initiation of treatment. According to Cal et al. (2009) a positive correlation was found between the severity of histological lesions and AST. Santos et al. (2011) observed elevation of the enzyme only in the animals that died, while the GGT was elevated in all conditions presented by the disease. Thus, increases in GGT were observed in sheep with PT by Wierda et al. (1985), who reported that serum GGT and sorbitol dehydrogenase activity are good metabolic indicators of PT prognosis.

The decrease in serum calcium concentration found in sheep and goats with PT was reported in the works of Sigurdsson (1991), Brozos et al. (2011), and Hefnawy et al. (2011), which reinforces the idea of the importance of calcium in the metabolism of sheep at the end of gestation and its possible involvement in the pathogenesis of PT. According to Schlumbohm et al. (1997), hypocalcemia associated with decreased insulin target tissue responsiveness, occurring during late gestation, worsens this condition through the inhibitory effect on hepatic gluconeogenesis and by increasing insulin resistance in target tissues. Schlumbohm & Harmeyer (2003) added that the combination of hypocalcemia and hyperketonemia has an additional effect in pregnant sheep on depression of the homeostatic glucose system. This information was strengthened by Schulla et al. (2003) who stated that insulin is secreted by pancreatic cells in response to elevation of cytoplasmic Ca^{2+} resulting from an increase in Ca^{2+} influx through the Ca^{2+} dependent voltage channels. This justifies the high positive relation found between these variables ($r=0.65$).

The chlorine and potassium elements presented an increase and decrease, respectively, in relation to the reference values for the ovine species, going against the findings of González et al. (2012), after induction and rapid intervention in goats under study, concluded that these electrolytes are not good initial indicators in the diagnosis of PT, as they do not present significant alteration, however, in natural cases, the intervention is usually later, which may have inferred in the results found. However, Henze et al. (1998) evaluated the spontaneous occurrence of PT and found a significant reduction in serum potassium levels in the sheep who died, attributing hypokalemia as a result of inadequate food intake and renal tubular absorption of potassium. According to Payne (1983), potassium plays a key role in certain enzymatic interactions,

especially in those related to energy metabolism; however it is difficult for potassium deficiency to occur as the majority of foods contain proportions higher than those required by animals. On the other hand, cereals are comparatively poorer than forage and the current feed system rich in concentrates, intended to increase the level of production, has altered this pattern.

The decrease found in serum insulin levels in sheep with PT was also observed by Sigurdsson (1991) and Henze et al. (1998) who found a more pronounced decrease in those animals in a more serious and life-threatening clinical condition. Alterations in insulin behavior were analyzed by Petterson et al. (1993), who found development of insulin resistance in peripheral tissues in sheep, using glucose as part of the adjustment of metabolic adaptations that occur at the end of gestation. According to Araujo (2009), maternal insulin-dependent tissues are placed at a competitive disadvantage by the pregnant uterus which is insulin independent, thus the constant drainage of maternal glucose into fetuses results in lower production of insulin by pancreatic cells.

Higher cortisol levels differ from those found by Firat & Özpınar (2002) in healthy sheep at the end of gestation. The authors observed a reduction in cortisol level concentrations at the end of gestation, but the increase was also noticed by Sigurdsson (1991) in cases of PT in sheep. According to Andrews (1997), serum cortisol levels are elevated as a result of reduced hepatic metabolism, hypoglycemia, and exposure to continuous levels of stress. According to Kimberling (1988), PT increases the size of the adrenal glands in response to increasing production of cortisol levels that may inhibit the use of glucose by tissues and cause clinical manifestations, especially associated with neurological signs. According to Reid (1960), this inhibitory effect on glucose utilization may be increased under conditions of severe insulin insufficiency. This fact was verified in the cases under study, in which there was a high negative correlation between cortisol and insulin ($r = -0.71$).

The macroscopic alterations found in the livers of the animals with PT are in agreement with those found by Kabakci et al. (2003), who in animals with good body condition, when necropsied, observed the liver with pale yellow coloration, increased in size and easily friable, suggesting extensive fatty infiltration. According to Andrews (1997), it varies from pale pink to yellowish orange, is often greasy to the touch and in some cases will float in water. The literature does not make any provisos about the differentiation of the macroscopic findings of livers between sheep with type I and type II PT, although there was a difference in this study.

Macroscopically, there were no significant alterations in the kidneys (Andrews 1997, Radostits et al. 2007), with only paleness of the surface and cortex (Parry & Taylor 1956) in some of the lean animals and slight yellowing in fatty animals.

In microscopy, massive lipid levels were observed in hepatocytes in spontaneous (Kabakci et al. 2003) and induced cases of PT (Cal et al. 2009). This alteration is related to the lipomobilization caused by the great energy demand due to gestation (Kabakci et al. 2003, Cal et al. 2009). This was also observed in the present study, especially in the sheep of Group 2.

Regarding the distribution of lesions, Cullen (2009) states that in mild cases the vacuolated hepatocytes are concentrated in the centrilobular region. This fact was not confirmed in the animals of Group 1, in which the lesions

in the periportal zone predominated (71.43%), unlike the centrilobular lesions (28.57%). However, the findings of Group 2 corroborate Cullen (2009), where the majority of the vacuolations were distributed throughout the three zones, reflecting a considerably enlarged organ with an extremely greasy texture.

The histopathology findings of the kidneys, as well as those of the liver, are associated with metabolic disorders, being similar to those found by Parry & Taylor (1956). These authors did not observe specific morphological alterations in the kidneys of the sheep with PT that died, except for unusual amounts of fat in the tubules, according to the findings of the disease in humans. These observations suggest that renal changes in sheep with PT could be purely functional and mainly reversible as in humans. It can be observed that there is a strong relation between body condition score and vacuolations in liver and kidney cells. Thus, the animals of Group 1 demonstrate scant vacuolization of hepatocytes, being little accompanied by vacuolization of cells of the renal epithelium.

It is interesting to mention the finding in one of the animals in Group 2, which presented unusual hepatocyte vacuolization and was necropsied two months after the diagnosis of gestation toxemia, with histological characteristics of the liver that returned to the initial pattern, as addressed by Cal et al. (2009) who observed this regenerative dynamic 60 days after PT induction, a fact corroborated by Parry & Taylor (1956) who noticed reversibility of lesions in the affected sheep's kidneys, supposing that in this animal cellular regeneration also occurred.

CONCLUSION

Metabolic disorders in sheep associated with hepatic and renal lesions are more apparent in overweight animals and with a predominance of vacuolization. With higher impairment of hepatic and renal function, the clinical prognosis of animals with a high body score can be considered reserved.

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Comparison of electrolyte and acid-base balances of Dorper breed ewes between single and twin pregnancies¹

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ABSTRACT.- Santarosa B.P., Dantas G.N., Ferreira D.O.L., Carvalho M.G., Rodrigues M., Pereira P.F.V., Silva A.A. & Gonçalves R.C. 2019. **Comparison of electrolyte and acid-base balances of Dorper breed ewes between single and twin pregnancies.** *Pesquisa Veterinária Brasileira* 39(10):789-795. Departamento de Clínica Veterinária, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Campus de Botucatu, Distrito de Rubião Júnior, Botucatu, SP 18618-970, Brazil. E-mail: biancasantarosavet@gmail.com

During pregnancy there are modifications in the metabolic profile of sheep that may predispose to the occurrence of metabolic disorders, of which pregnancy toxemia (PT) is highlighted. Blood gas analysis detects changes in acid-base and electrolyte balance effectively. The objectives of this study were to study the acid-base and electrolyte balance of sheep during gestation and in the immediate peripartum (up to 48 hours postpartum), comparing single gestation with twins. Sixty healthy sheep of Dorper breed, two to five years old were raised in a semi-intensive system and were divided in two experimental groups: Group 1: 30 ewes, with ultrasonographic diagnosis of single fetus gestation; Group 2: 30 ewes, with ultrasonographic diagnosis of twin pregnancy. The experimental moments were defined as: MI-immediately after artificial insemination (control); MG30 - 30 days of gestation; MG90 - 90 days of gestation; MG120 - 120 days of gestation; MG130 - 130 days of gestation; MG140 - 140 days of gestation; MP - lambing; MPP1 - 24h postpartum; MPP2 - 48h postpartum. At all times 1mL of blood was collected per jugular vein puncture for blood gas evaluation in a portable equipment (I-Stat[®]). The pH, carbon dioxide pressure (PCO₂), bicarbonate (HCO₃⁻), base excess (BE), sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), ionized calcium (Ca⁺²), lactate, anion gap (AG) were measured and strong ion difference (SID) were calculated. This work showed that there were changes in acid-base and electrolyte balance in pregnant ewes, due to the decrease in BE, HCO₃⁻, TCO₂ and increase of lactate and AG during gestation, but the pH remained normal and did not present any difference among moments in both groups. Comparing the groups, single-gestation ewes presented higher alkaline expenditure at delivery than twin-gestation, evidenced by lower levels of BE and HCO₃⁻. Lower Na⁺ levels were observed in prepartum; drop in K⁺ values with advancing gestation; hyperchloremia and hypocalcemia during gestation according to the reference standards for

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species; decreased iCa^{+2} levels in the final third of gestation in both groups. It was concluded that the ewes of this study were healthy until the last moment analyzed (48h postpartum), although have shown greater consumption of the alkaline reserve in the peripartum, being more explicit in the single gestation animals. In addition, this situation can be physiological and result in increased energy demand during gestation, more exacerbated by peripartum.

INDEX TERMS: Electrolyte, acid-base, Dorper breed, ewes, twin pregnancies, blood gas analysis, metabolic diseases, pregnancy, small ruminants, sheep, ovine.

RESUMO.- [Comparação dos equilíbrios eletrolítico e ácido básico de ovelhas da raça Dorper entre gestação única e gemelar.]

Durante a gestação ocorrem modificações metabólicas nas ovelhas que podem predispor a ocorrência de transtornos metabólicos, dos quais se destaca a toxemia da prenhez (TP). A hemogasometria é um exame que detecta alterações nos equilíbrios ácido-básico e eletrolítico de forma eficaz. Os objetivos deste estudo foram estudar os equilíbrios ácido-básico e eletrolítico de ovelhas durante a gestação e no periparto imediato (até 48 horas pós-parto), comparando-se gestação única com gemelar. Foram utilizadas 60 ovelhas criadas em manejo semi-intensivo, híbridas, da raça Dorper, com dois a cinco anos de idade. Foram constituídos dois grupos experimentais: Grupo 1: 30 ovelhas, com diagnóstico ultrassonográfico de gestação de feto único; Grupo 2: 30 ovelhas, com diagnóstico ultrassonográfico de gestação gemelar. Os momentos experimentais foram definidos como: MI - imediatamente após a inseminação artificial (controle); MG30 - 30 dias de gestação; MG90 - 90 dias de gestação; MG120 - 120 dias de gestação; MG130 - 130 dias de gestação; MG140 - 140 dias de gestação; MP - dia do parto; MPP1 - 24h pós-parto; MPP2 - 48h pós-parto. Em todos os momentos foi colhido 1mL de sangue por punção da veia jugular para avaliação hemogasométrica em aparelho portátil (I-Stat®). Foram analisados os parâmetros: pH, pressão de dióxido de carbono (PCO_2), bicarbonato (HCO_3^-), excesso de bases (EB), sódio (Na^+), potássio (K^+), cloreto (Cl^-), cálcio ionizado (iCa^{+2}), lactato, ânion gap (AG) e diferença de íons fortes (SID). Este trabalho mostrou que houve mudanças nos equilíbrios ácido-básico e eletrolítico nas ovelhas prenhes, pela diminuição do EB, HCO_3^- e aumento do lactato e AG no decorrer da gestação, porém o pH se manteve dentro da normalidade e não apresentou diferença ao longo dos momentos em ambos os grupos. Comparando os grupos, as ovelhas de gestação única apresentaram maior consumo da reserva alcalina no momento do parto do que as de gestação gemelar, evidenciado menores níveis de EB e HCO_3^- . Foram observados níveis mais baixos de Na^+ no pré-parto; queda dos valores de K^+ com o avanço da gestação; hipercloremia e hipocalcemia durante a gestação segundo os padrões de referência para espécie e diminuição dos níveis de iCa^{+2} no terço final da gestação nas ovelhas de ambos os grupos. Concluiu-se que as ovelhas deste estudo apresentaram-se saudáveis até o último momento analisado (48h pós-parto), embora tenham mostrado maior consumo da reserva alcalina no periparto, sendo mais evidente nos animais de gestação única. Apesar disso, essa ocorrência pode ser considerada fisiológica e consequência do aumento da demanda energética durante a gestação, mais exacerbada no periparto.

TERMOS DE INDEXAÇÃO: Equilíbrio eletrolítico, ácido básico, ovelhas, raça Dorper, gestação única, gestação gemelar doenças metabólicas, gestação, hemogasometria, pequenos ruminantes, ovinocultura, ovinos.

INTRODUCTION

The gestational period involves several anatomical, hormonal, and metabolic changes due to the preparation for delivery and early lactation. These changes may cause metabolic disorders such as pregnancy toxemia (PT), which affects ewes and goats in the third and final period of pregnancy (Campos et al. 2010, Souto et al. 2013, Souza et al. 2016).

Santos et al. (2011) studied PT in ewes and found metabolic acidosis explained by ketonemia due to excessive production of ketone bodies (acetoacetate, acetone, and beta-hydroxybutyrate), which is a consequence of lipoxidation. As these compounds have acidic characteristics, they increase the anion gap (AG) and reduce the concentration of bicarbonate (HCO_3^-), chloride (Cl^-), sodium (Na^+) and potassium (K^+). In addition, sheep and goats may present hypocalcemia and hypomagnesemia (Souto et al. 2013).

Blood gas analysis is a laboratory test that analyzes blood gases and acid-base balance quickly and conveniently. Many acid-base balance disorders bring about few disorders in the body because they are transient or properly compensated by the animal. However, in some situations, correcting this metabolic profile is essential for the individual's survival. Thus, the blood gas analysis provides data to inform diagnosis, help treatment, and establish a prognosis of several ruminant diseases such as PT (Ortolani 2003). Base excess values, for instance, are used to calculate the volume of bicarbonate to be infused in an animal with metabolic acidosis (Cosenza et al. 2015). González et al. (2012) found a decrease in blood pH, HCO_3^- , and base excess (BE), in addition to an increase in the anion gap [$(Na^+ + K^+) - (HCO_3^- + Cl^-)$] in goats experimentally induced PT, indicating that such parameters may identify early cases of the disease.

The acid-base status of extracellular fluid is affected directly by the concentration of strong cations (Na^+ e K^+) and strong anions (Cl^- , S^{2-} and lactate). Measuring electrolytes such as Na^+ , K^+ e Cl^- to calculate strong ion difference ($SID = [Na^+ + K^+] - Cl^-$) provides information on acid-base equilibrium, highlighting the influence of electrolytes on it (Constable 1999, 2003). The increase in plasma SID can be produced by increasing Na^+ and K^+ and/or decreasing Cl^- . According to this approach, this would result in increased pH and HCO_3^- - variables considered dependent - thus creating an alkalinizing effect. The reduction in plasma SID has the opposite result, generating an acidifying effect (De Moraes & Constable 2006).

Although many studies discuss the clinical and laboratory findings of sheep with PT, there are no studies in the literature analyzing the metabolic profile of sheep from conception to parturition and postpartum to identify the physiological processes of pregnancy in the acid-base and electrolyte balance, and subsequently to recognize changes that help inform early diagnosis of the disease. The aim of this study was

to analyze acid-base and electrolyte balance in Dorper ewes during pregnancy, parturition, and immediately postpartum, comparing single and twin pregnancies.

MATERIALS AND METHODS

The methods used in this study were approved by the Animal Research Ethics Committee (CEUA) of "Faculdade de Medicina Veterinária e Zootecnia" (FMVZ) of "Universidade Estadual Paulista" (Unesp), Botucatu Campus (Protocol 189/2014).

Healthy, hybrid Dorper ewes aged two to five years old that participated in an estrus synchronization protocol were selected. The long-term protocol (12 days) was used, in which the 0.33g progesterone vaginal implant (Cidr®, Zoetis) was placed on day 0 and kept for 12 days (D0 to D12). It was removed after this period, when 400 to 500IU of equine chorionic gonadotropin (eCG) (Novormon®, Zoetis) were intramuscularly administered (Bicudo & Sousa 2002) to each animal. After 48 hours (D14), fixed-time artificial insemination (FTAI) with frozen semen was performed by laparoscopy, and the first moment, considered as control moment (MI), was collected. After 30 days (MG30), the diagnosis of single or twin pregnancy (Santos et al. 2004) was performed with a portable ultrasound device (My LabTM30 Vet Gold Esaote®, Esaote Healthcare Brazil, São Paulo/SP, Brazil) with a 5.0MHz linear transducer for transrectal examination upon defining the experimental group in which the ewes were included.

Two experimental groups were created: Group 1, which included 30 ewes with an ultrasound diagnosis of single fetal pregnancy, and Group 2, which included 30 ewes with an ultrasound diagnosis of twin pregnancy. Empty animals were excluded from the experimental groups. For the other evaluation moments, a transabdominal ultrasound was performed with a 3.5 MHz convex transducer (Santos et al. 2004) to assess fetal viability. All ewes received deworming after parturition (Monepantel, Zolvix®, Novartis Animal Health) and multipurpose vaccine against Clostridiosis (Symxan-T Polyvalent®, Merial) at 30 and 60 days before parturition.

The ewes were released in the morning and grazed during the day. The pasture used was Vaquero grass (cultivar *Cynodon dactylon*). In the late afternoon, the animals were enclosed in 12m² collective pens in a masonry shed with rice straw bed, where they were fed with 0.5 kg/animal of maintenance feed and 1.0kg/animal of corn silage. In the shed, commercial mineral salt for ewes was available *ad libitum* (Ovinofós com Monensina®, Tortuga Companhia Zootécnica Agrária, Mairinque/SP, Brazil) in covered masonry troughs. The water came from an artesian well located in the farm, available *ad libitum* from automatic troughs. The total diet of the ewes was composed of pasture, corn silage, and feed made on the property based on corn meal, soybean meal, urea, mineral salt, and commercial seeds.

Experimental moments were defined as MI = immediately after FTAI (control); MG30 = 30 days of gestation; MG90 = 90 days of gestation; MG120 = 120 days of gestation; MG130 = 130 days of gestation; MG140 = 140 days of gestation; MP = day of delivery; MPP1 = 24 hours after delivery; MPP2 = 48 hours after delivery. All of the blood samples were collected by jugular vein puncture in the morning at the nine moments having the animals from both experimental groups been fasting. Blood was collected (1mL) in a polyethylene syringe heparinized with sodium heparin (Hemofol®, Cristália Pharmaceuticals Ltda., São Paulo/SP) fitted with a 30x8mm needle (BD®, BD Medical, Curitiba/PR, Brazil) and subsequently sealed with rubber. Immediately after collection, a blood gas analysis was performed on a portable pH, electrolyte, and blood gas analyzer (I-Stat®, Abbott Laboratories, Illinois, USA). CG4+ and CHEM8+

cartridges were used (I-STAT®, Abbott Laboratories, Illinois, USA). The CG4+ cartridge measured the values of pH, carbon dioxide pressure (PCO₂), oxygen pressure (PO₂), and lactate for calculation of the values of HCO₃⁻, TCO₂, BE, and oxygen saturation (sO₂). The CHEM8+ cartridge measured sodium (Na⁺), potassium (K⁺), chloride (Cl⁻), ionized calcium (iCa²⁺), and calculated the anion gap (AG). The Strong Ion Difference values [SID = (Na⁺ + K⁺) - Cl⁻] (Constable 1999) were also calculated.

The statistical analysis was performed using the SigmaStat 3.5 software. For all parameters of blood gas analysis, except BE, a One-Way Repeated Measures ANOVA was performed to compare the evaluation moments within each group individually. When significance (P<0.05) was found, the Tukey test was performed to compare the means (P<0.05). At each moment, Student's t-test was also performed to compare the study groups (P<0.05). As they do not present a normal distribution, the nonparametric analysis was used for the data concerning BE. The Friedman test was performed with repeated measures to compare the evaluation moments within each group individually. When significance (P<0.05) was found, the Dunn test was used to compare the medians (P<0.05). At each moment, the Mann-Whitney test was also performed to compare the groups under study (P<0.05).

RESULTS AND DISCUSSION

None of the ewes presented clinical symptoms suggesting PT or any other disease. Thus, no treatment was administered in the property where the samples were collected. Only one sheep with a simple gestation required a cesarean section due to dystocia of maternal origin caused by failure of cervical dilation. Fifty-one out of the 59 normal deliveries were assisted, where fetal dystocia was the most common cause. The mean and standard deviation of the gestational period of the 60 ewes was 144.75±2.12 days of gestation, ranging from 140 to 149 days. The average birth weight of the 90 lambs was 3.29±0.94kg, with 42 females and 48 males. Most ewes calved at dawn or dusk, with only five sheep calving in the pasture in the daytime.

The blood pH (Table 1) of the ewes showed no difference between the moments and groups, and all the means were within the reference values for the species. Therefore, the animals remained healthy throughout their pregnancy, although changes were detected during the pregnancies in other acid-base balance parameters. In contrast with this study, Khatun et al. (2011) found alkaline blood pH in ewes at five stages of pregnancy, in addition to describing a difference between the first (14 to 57 days) and the last periods (121 to 140 days) analyzed, although no explanation was found for the fact.

The mean PCO₂ values (Table 1) of the ewes in this study were within the normal range for the species in both groups and at all times. There was a difference between the groups in two moments, with the mean of G1 higher than G2 in MG30 and MG120. The highest values in both groups were found in MI, which may be explained by the greater agitation of the animals during administration of FTAI, presenting hypercapnia compared to the other moments, when the sheep had already gotten used to collection. The lowest PCO₂ levels were found in MP for G1 and MG120 for G2; however, no clinical significance was found.

A difference in the BE medians (Table 2) was only found between the groups in MP, when the twin pregnancy group

Table 1. Venous blood pH and concentration of PCO₂ (mmHg) in the ewes included the two experimental groups (G1 and G2) at different times (M) of collection

M	pH		P	PCO ₂		P
	G1	G2		G1	G2	
MI	7.38 ± 0.08	7.40 ± 0.05	0.439	41.4 ± 5.3 ^a	42.2 ± 3.9 ^a	0.580
MG30	7.38 ± 0.03	7.39 ± 0.04	0.292	38.2 ± 2.9 ^{abcA}	36.4 ± 2.7 ^{bcB}	0.025
MG90	7.37 ± 0.07	7.40 ± 0.04	0.109	40.6 ± 7.0 ^{ab}	37.8 ± 4.0 ^{bc}	0.105
MG120	7.39 ± 0.06	7.40 ± 0.04	0.402	38.6 ± 5.4 ^{abcA}	34.8 ± 3.7 ^{cB}	0.007
MG130	7.37 ± 0.05	7.39 ± 0.03	0.285	37.1 ± 4.4 ^{bc}	35.7 ± 3.8 ^{bc}	0.251
MG140	7.39 ± 0.08	7.39 ± 0.04	0.831	37.3 ± 5.7 ^{bc}	36.5 ± 4.5 ^{bc}	0.566
MP	7.35 ± 0.08	7.38 ± 0.05	0.094	36.2 ± 4.6 ^c	37.1 ± 4.1 ^{bc}	0.441
MPP1	7.37 ± 0.07	7.38 ± 0.06	0.418	37.8 ± 5.4 ^{abc}	37.9 ± 5.3 ^{bc}	0.931
MPP2	7.38 ± 0.08	7.37 ± 0.07	0.610	37.8 ± 4.0 ^{abc}	38.5 ± 3.1 ^b	0.515
P	0.195	0.651		<0.001	<0.001	

Data are shown as mean ± standard deviation; ^{a,b,c} Means followed by the same lower case letter in the columns did not differ statistically by the Tukey test (P>0.05); ^{A,B} Means followed by different upper case letters in the rows differed statistically by the t-test (P<0.05); blood pH = 7.32 to 7.54 (Kaneko et al. 2008), 7.28 to 7.42 (Ortolani 2003), PCO₂ = 34 to 45mmol/L (Ortolani 2003).

Table 2. Venous blood concentration of BE (mmol/L) in the ewes included in the two experimental groups (G1 and G2) at different times (M) of collection

M	BE		P
	G1	G2	
MI	1.0 (-3.0 - 2.2) ^a	1.0 (-2.0 - 3.0) ^a	0.467
MG30	-2.0 (-3.0 - -1.0) ^{ab}	-3.0 (-5.0 - 1.0) ^{ab}	0.300
MG90	-2.0 (-2.0 - -4.0) ^{abc}	-2.0 (-3.0 - 0.0) ^{ab}	0.495
MG120	-1.0 (-1.0 - -5.0) ^{ab}	-4.0 (-5.0 - -1.0) ^b	0.174
MG130	-4.0 (-4.0 - -5.0) ^{bc}	-4.0 (-5.0 - -2.0) ^b	0.908
MG140	-3.0 (-3.0 - -5.0) ^{abc}	-3.5 (-5.0 - 0.0) ^b	0.834
MP	-5.0 (-5.0 - -7.0) ^{cB}	-4.0 (-5.0 - -2.0) ^{bA}	0.046
MPP1	-3.0 (-3.0 - -6.0) ^{bc}	-2.5 (-6.0 - 0.0) ^{ab}	0.480
MPP2	-2.0 (-2.0 - -5.0) ^{abc}	-2.0 (-5.0 - 1.0) ^{ab}	0.939
P	<0.001	<0.001	

Data are shown as medians, first and third percentiles; ^{a,b,c} Medians followed by the same lower case letter in the columns did not differ statistically by Dunn's test (P>0.05); ^{A,B} Medians followed by different upper case letters in the rows differed statistically by the Mann-Whitney test (P<0.05); BE = -4 to 2mmol/L (Ortolani 2003).

presented lower base excess compared to the simple gestation group. Only the MP median in G1 was below the normal range for the species, according to Ortolani (2003). In both groups, the highest values were observed in MI, when the ewes were still empty, and the lowest results were found in MP. This finding represented a consumption of alkaline reserve during delivery, when ketone bodies were probably produced and, therefore, with compensated metabolic acidosis due to the acid character of the circulating ketone bodies, given the lower food intake due to the increase in the volume of the pregnant uterus (Santos et al. 2011). However, moments MG120, MG130, and MG140 were equal to MP in G2. This may be justified by the fact that twin-gestated ewes are already subject to a higher energy demand from the last month of gestation, due to the greater development of two lambs, and thus already develop ketonemia and, consequently, present a base deficit (Santos et al. 2011).

The HCO₃⁻ means (Table 3) of G1 were higher than those of G2 in MG120; in MP, the opposite occurred, which also coincided with the BE values. In both groups and parameters, the highest means were observed in MI, whereas the lowest were observed in MP. Group 1 presented a greater variation between the moments, while in G2 only MI differed from the others. Although there were differences across moments, all means were within the normal range for the species, according to Ortolani (2003), which shows changes in metabolism during pregnancy that can be considered physiological.

The lactate means (Table 3) were different between the groups in the MI and MG130 moments, where the ewes in G1 presented higher values than those in G2. In MG30, i.e. the first moment of analysis of the gestational period, the lowest values were found in both groups. It was also the only moment with results within the normal range for the species. In MP, the mean lactate values were higher than in the other moments analyzed. As in other species such as human beings, horses, and cattle this elevation during parturition in ewes is physiological and due to the generalized stimulation of the sympathetic nervous system with adrenaline and cortisol release during and after birth, promoting the increase of lactate and globular volume, glucose, and free fatty acids (Comline & Silver 1972). Similarly to this study, Silva et al. (2013) reported higher lactate levels in Suffolk crossbred ewes at the time of eutocic parturition (3.0±0.5mmol/L).

The ewes of both groups presented hyperlactatemia during MI when experiencing stress during administration of FTAI and throughout their whole pregnancy and postpartum, except for MG30. Santos et al. (2011) described high lactate levels in natural cases of PT in ewes and attributed it to ketonemia leading to metabolic acidosis, in addition to associating the glycolytic process (anaerobic glucose), which produces lactate from glucose, contributing to hyperlactatemia. In the absence of clinical manifestation of PT in this study, high lactate levels were probably related to a high energy demand, which promoted the more exacerbated production of this component, besides physiological elevation during delivery.

The groups differed in MG140 with regard to the Na⁺ dosage (Table 4), where G2 was greater than G1. All means were within the normal range for the species. Overall, the means were

Table 3. Venous blood concentrations of HCO_3^- and lactate (mmol/L) in the ewes included in the two experimental groups (G1 and G2) at different times (M) of collection

M	HCO_3^-			Lactate		
	G1	G2	P	G1	G2	P
MI	24.4 ± 3.5 ^a	25.6 ± 2.6 ^a	0.196	3.1 ± 2.9 ^{bcA}	1.7 ± 1.1 ^{cdB}	0.033
MG30	22.4 ± 1.8 ^{ab}	21.9 ± 2.2 ^b	0.348	1.0 ± 0.7 ^d	0.8 ± 0.5 ^d	0.208
MG90	22.7 ± 2.1 ^{ab}	22.7 ± 2.0 ^b	0.980	2.3 ± 1.9 ^{bcd}	1.8 ± 1.5 ^{bcd}	0.393
MG120	22.7 ± 2.3 ^{abA}	21.2 ± 2.7 ^{bB}	0.035	1.9 ± 1.7 ^{cd}	1.5 ± 1.0 ^{cd}	0.396
MG130	21.2 ± 2.0 ^{bc}	21.2 ± 2.5 ^b	0.940	2.1 ± 1.4 ^{bcdA}	1.4 ± 0.6 ^{cdB}	0.045
MG140	21.9 ± 2.9 ^b	21.8 ± 2.8 ^b	0.862	2.2 ± 2.8 ^{bcd}	1.4 ± 0.9 ^{cd}	0.185
MP	19.4 ± 3.2 ^{cB}	21.6 ± 3.0 ^{bA}	0.016	5.3 ± 4.2 ^a	3.3 ± 2.5 ^a	0.060
MPP1	21.2 ± 3.2 ^{bc}	22.1 ± 2.9 ^b	0.345	3.9 ± 2.8 ^{ab}	3.0 ± 2.0 ^{ab}	0.214
MPP2	22.2 ± 3.2 ^b	22.2 ± 3.7 ^b	0.980	2.4 ± 1.6 ^{bcd}	2.5 ± 1.7 ^{abc}	0.841
P	<0.001	<0.001		<0.001	<0.001	

Data are shown as mean ± standard deviation; ^{a,b,c,d} Means followed by the same lower case letter in the columns did not differ statistically by the Tukey test ($P>0.05$); ^{A,B} Means followed by different upper case letters in the row differed statistically by the t-test ($P<0.05$); HCO_3^- = 20 to 25mmol/L (Kaneko et al. 2008), 19 to 25 mmol/L (Ortolani 2003), Lactate = 1 to 1.33mmol/L (Kaneko et al. 2008).

Table 4. Venous blood concentrations of Na^+ and K^+ (mmol/L) in the ewes included in the two experimental groups (G1 and G2) at different times of collection

M	Na^+			K^+		
	G1	G2	P	G1	G2	P
MI	143.6 ± 2.8 ^e	144.7 ± 1.5 ^d	0.088	5.0 ± 1.9 ^a	4.5 ± 0.9 ^a	0.269
MG30	146.1 ± 2.0 ^d	146.1 ± 1.8 ^d	0.967	4.0 ± 0.4 ^{bc}	3.8 ± 0.3 ^{bc}	0.106
MG90	145.1 ± 1.8 ^{de}	145.3 ± 1.5 ^d	0.597	4.4 ± 1.0 ^{ab}	4.1 ± 0.3 ^b	0.175
MG120	147.7 ± 1.5 ^c	147.9 ± 1.5 ^c	0.667	3.9 ± 0.4 ^{bc}	3.8 ± 0.3 ^{bc}	0.281
MG130	150.5 ± 2.1 ^a	150.5 ± 2.3 ^a	0.918	4.0 ± 0.5 ^{bcA}	3.7 ± 0.3 ^{cB}	0.003
MG140	147.7 ± 1.1 ^{cB}	148.7 ± 2.0 ^{cA}	0.039	3.9 ± 0.4 ^{bcA}	3.7 ± 0.3 ^{cB}	0.012
MP	149.2 ± 2.2 ^{abc}	150.4 ± 2.6 ^{ab}	0.077	3.8 ± 0.3 ^{bcA}	3.6 ± 0.4 ^{cB}	0.035
MPP1	149.6 ± 1.7 ^{ab}	149.0 ± 2.1 ^{bc}	0.219	3.6 ± 0.4 ^c	3.8 ± 0.4 ^{bc}	0.106
MPP2	148.8 ± 1.7 ^{bc}	149.0 ± 1.3 ^{abc}	0.699	3.6 ± 0.3 ^c	3.7 ± 0.4 ^c	0.687
P	<0.001	<0.001		<0.001	<0.001	

Data are shown as mean ± standard deviation; ^{a,b,c,d,e} Means followed by the same lower case letter in the columns did not differ statistically by the Tukey test ($P>0.05$); ^{A,B} Means followed by different upper case letters in the rows differed statistically by the t-test ($P<0.05$); Na^+ = 139 to 152mmol/L, K^+ = 3.9 to 5.4mmol/L (Kaneko et al. 2008).

equal in both groups from MI to MG90, increased in MG130 (higher values), decreased in MG140, and presented higher values again in MP. As in this study, Azab & Abdel-Maksoud (1999) noted lower Na^+ levels in goats one week before calving. This fact may be justified by lower peripartum food intake and great loss of body fluids at delivery. Additionally, the aqueous fraction of colostrum is mainly composed of Na^+ , K^+ , and Cl^- ions, and its production occurs seven days before delivery (Azab & Abdel-Maksoud 1999).

The means of K^+ (Table 4) at the MG130, MG140, and MP moments were different between the groups, where G1 was greater than G2. The potassium means declined from the time the ewes were empty (MI) to the first moment of pregnancy analyzed (MG30) and continued to fall until the postpartum moments. The same situation was observed by Azab & Abdel-Maksoud (1999), who analyzed the electrolyte balance of goats four weeks before and after delivery. Decreased food intake due to increased uterine volume may lead to hypokalemia. Regarding the normality pattern for sheep, the ewes were hypokalemic from MP to MPP2 in G1, and in MG30 and from MG120 to MPP2 in G2.

There was a statistical difference between groups for the measurement of Cl^- (Table 5) only in MG120, in which G2 was greater than G1. All means of both groups showed that the ewes presented hyperchloremia, according to the normality standards proposed by Kaneko et al. (2008). The highest values were found in MG130, whereas the lowest values were found in MG90 for both groups. In concentrations close to the upper limit in this study, hyperchloremia may not necessarily be related to pregnancy, since the mean values of Cl^- in the empty ewes were also slightly above reference; therefore, it cannot be considered clinically significant. As in this study, Cosenza et al. (2013) described the mean value of 116.46 ± 1.88 mmol/L chloride in healthy, but non-pregnant or lactating ewes. The chloride levels did not mean a pathological process, but could be linked to the mineral salt intake of the ewes during pregnancy (Souza et al. 2016), which occurred *ad libitum* from the salt trough and was added to the feed. Souza et al. (2016) described reference values for Dorper ewe electrolytes in Bahia considering gender and age, showing that the lower limit of chloride values of animals aged 12 to 24 months (73,57-142,37mg/dL) was higher than

those of young animals from one to six months, due to the higher consumption of mineral salt in this age group, although without statistical difference.

With regard to iCa^{+2} (Table 5), no difference was found between groups at any of the moments analyzed. A similar behavior was found in both groups in the different moments, with the highest values observed in MG90, the lowest in MI, and similar values in the others. However, all means were below normal for the species, although the ewes showed no clinical signs of hypocalcemia. Overall, the ewes presented increased calcium levels from MI to MG30, as well as from MG30 to MG90, and decreased values from MG90 to MG120, which remained the same until MPP2. This decrease in calcium levels may be related to the higher demand of the last month of pregnancy due to fetal mineralization and the production of the maternal colon, which requires a considerable amount of circulating levels of this element (Souto et al. 2013).

The mean values of AG (Table 6) of the groups only differed in MG120, being greater in G1 compared to G2. Additionally, in both groups, the MI and MG30 moments were equal. The results increased from MG90 until the moment of delivery

(MP), remaining the same until 48h postpartum. During this period from MG90 to MPP2 the AG mean of the pregnant ewes in both groups showed metabolic acidosis, however compensated, since the blood pH remained unchanged.

The SID values (Table 6) were lower at the first moment of pregnancy analysis (MG30), whereas the highest values were found 48h postpartum (MPP2) for the ewes in both groups. A priori, this fact can be explained by the adaptation of the maternal organism to the gestation observed at 30 days. In the MPP2 moment, the metabolic normalization in the acid-base imbalance observed in the peripartum was observed and confirmed, albeit physiologically, by the bicarbonate and base excess levels (Table 2). A slight decrease in pre-partum SID values from MG130 to MG140 was observed. This represents a decrease below the reference values for the species and the healthy ewe data described by other authors. Ferreira et al. (2014) studied the influence of ammonium chloride supplementation in confined lambs and described that animals in the control group (not supplemented) presented mean SID values between 41.78 ± 1.56 and 44.46 ± 1.69 mEq/L. On the other hand, Conseza et al. (2015), in a study on acute ruminal

Table 5. Venous blood concentrations of Cl^- and iCa^{+2} (mmol/L) in the ewes included in the two experimental groups (G1 and G2) at different harvest times

M	Cl^-			iCa^{+2}		
	G1	G2	P	G1	G2	P
MI	109.2 ± 3.2^{cd}	108.8 ± 2.7^{cd}	0.612	0.72 ± 0.10^c	0.76 ± 0.11^c	0.202
MG30	111.4 ± 2.2^{abc}	111.7 ± 2.6^{ab}	0.652	0.91 ± 0.10^b	0.91 ± 0.10^b	0.928
MG90	107.3 ± 2.4^d	108.1 ± 2.5^d	0.268	1.04 ± 0.13^a	1.05 ± 0.16^a	0.869
MG120	110.5 ± 2.6^{bcB}	112.6 ± 3.0^{abA}	0.009	0.90 ± 0.13^b	0.86 ± 0.11^{bc}	0.291
MG130	113.0 ± 2.6^a	113.4 ± 2.4^a	0.529	0.91 ± 0.15^b	0.85 ± 0.14^{bc}	0.117
MG140	111.2 ± 2.7^{abc}	112.8 ± 3.4^b	0.067	0.87 ± 0.09^b	0.88 ± 0.18^b	0.849
MP	112.5 ± 2.9^{ab}	112.3 ± 3.7^{ab}	0.792	0.91 ± 0.12^b	0.88 ± 0.14^b	0.424
MPP1	112.1 ± 2.9^{ab}	111.2 ± 3.1^{ab}	0.263	0.85 ± 0.15^b	0.89 ± 0.11^b	0.350
MPP2	110.4 ± 4.5^c	110.5 ± 2.7^{bcd}	0.904	0.90 ± 0.13^b	0.93 ± 0.11^{ab}	0.346
P	<0.001	<0.001		<0.001	<0.001	

Data are shown as mean $\pm$ standard deviation; ^{a,b,c,d} Means followed by the same lower case letter in the columns did not differ statistically by the Tukey test ($P > 0.05$); ^{A,B} Means followed by different upper case letters in the rows differed statistically by the t-test ($P < 0.05$); Cl^- = 95 to 103 mmol/L, iCa^{+2} = 1.4 to 1.6 mmol/L (Kaneko et al. 2008).

Table 6. Venous blood concentrations of AG (mEq/L) and SID (mmol/L) in the ewes included in the two experimental groups (G1 and G2) at different times (M) of collection

M	AG			SID		
	G1	G2	P	G1	G2	P
MI	18.3 ± 3.5^e	18.0 ± 2.0^e	0.789	39.4 ± 3.4^{bc}	40.4 ± 3.1^{abcd}	0.254
MG30	18.9 ± 1.3^e	19.1 ± 1.6^{de}	0.640	38.7 ± 2.2^c	38.2 ± 2.2^d	0.467
MG90	21.6 ± 3.7^{bcd}	21.3 ± 2.1^{bc}	0.712	42.2 ± 2.6^a	41.4 ± 2.8^{abc}	0.278
MG120	20.9 ± 2.3^{dA}	19.3 ± 1.8^{deB}	0.009	41.1 ± 2.7^{abA}	39.0 ± 2.9^{cdB}	0.011
MG130	22.9 ± 1.9^{bcd}	22.3 ± 2.4^{abc}	0.373	41.5 ± 2.8^b	40.8 ± 3.4^{abc}	0.392
MG140	21.1 ± 1.8^{cd}	20.5 ± 1.4^{cd}	0.267	40.5 ± 2.9^{abc}	39.6 ± 3.1^{bcd}	0.301
MP	24.1 ± 2.8^a	23.3 ± 2.9^a	0.317	40.5 ± 1.8^{abc}	41.8 ± 2.7^{ab}	0.056
MPP1	23.0 ± 2.1^{abc}	22.6 ± 1.9^{ab}	0.571	41.1 ± 2.8^{ab}	41.6 ± 1.9^{abc}	0.488
MPP2	23.0 ± 3.1^{ab}	23.3 ± 1.5^a	0.966	42.1 ± 4.1^a	42.2 ± 2.5^a	0.932
P	<0.001	<0.001		<0.001	<0.001	

Data are shown as mean $\pm$ standard deviation; ^{a,b,c,d,e} Means followed by the same lower case letter in the columns did not differ statistically by the Tukey test ($P > 0.05$); ^{A,B} Means followed by different upper case letters in the rows differed statistically by the t-test ($P < 0.05$); AG = 10 to 20 mmol/L, SID = 47.9 to 54.4 mmol/L (Kaneko et al. 2008).

lactic acidosis, described a mean SID value of 34.5 ± 4.04 mEq/L in empty and non-lactating ewes, which proved metabolic acidosis. Contrarily, this study found 38.2 ± 2.2 mEq/L as the lowest mean value, where the absence of clinical signs was compatible with the acid-base imbalance.

The blood gas analysis in this experiment showed that pregnancy caused higher alkaline reserve expenditure during delivery (MP), elucidated by BE values (Table 2), with decreased SID (Table 6). However, no metabolic acidosis was observed. Since there was no acidemia (Table 2), the events occurred physiologically, and the ewes were healthy and did not present pregnancy toxemia.

CONCLUSIONS

The ewes of this study were healthy until the last moment analyzed (48h postpartum), although they showed higher consumption of alkaline reserve in the peripartum, which was more evident in animals with single gestation. Nevertheless, this occurrence can be considered physiological and a consequence of the increased energy demand during pregnancy, which is more exacerbated in the peripartum.

The electrolytic changes observed across the moments were also considered physiological.

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Bacteria isolated from the lower respiratory tract of sheep and their relationship to clinical signs of sheep respiratory disease¹

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ABSTRACT.- Franco M.F., Gaeta N.C., Alemán M.A.R., Mellville P.A., Timenetsky J., Balara M.F.A. & Gregory L. 2019. **Bacteria detected from the lower respiratory tract of sheep and their relationship to clinical signs of sheep respiratory disease.** *Pesquisa Veterinária Brasileira* 39(10):796-801. Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva 87, Cidade Universitária, São Paulo, SP 05508-270, Brazil. E-mail: natalia.gaeta@hotmail.com

Respiratory diseases are among the most important diseases in sheep flocks. Herein was studied the bacterial etiology of respiratory disease and the clinical signs of 99 female and male sheep breed in the states of São Paulo (SP) and Rio de Janeiro (RJ), Brazil. After physical examination of animals, tracheobronchial flushing samples were obtained. The usual bacteria and *Mycoplasma* spp. were searched, as well as their association with the clinical status and clinical signs of sheep with respiratory disease. The main observed signs were: tachypnea (75%), increase of rectal temperature (09.4%), mucopurulent/purulent nasal discharge (21.9%), cough (25%), dyspnea (31.2%), changes of lung sounds at auscultation (87.5%) and chest percussion (28.1%) in pneumonic sheep. Non-fermenting gram-negative bacteria and *Bacillus* sp. were the most isolated bacteria. Microorganisms of the Mollicutes class were molecularly (PCR) detected in 33.3% of the animals. In addition, the specific detection of *M. mycoides* subsp. *capri* was described for the first time in sheep from the state of São Paulo, Brazil.

INDEX TERMS: Bacteria, lower respiratory tract, sheep, clinics, sheep respiratory disease, *Mycoplasma* spp., pneumonia, respiratory disease, bacterioses.

RESUMO.- [Bactérias detectadas no trato respiratório inferior de ovinos e a relação com as manifestações clínicas da doença respiratória ovina.] A doença respiratória é uma das doenças mais importantes em rebanhos ovinos. Esta pesquisa teve como objetivo determinar a etiologia bacteriana

da doença respiratória e sua relação com sinais clínicos em ovinos criados nos estados de São Paulo e Rio de Janeiro, Brasil. Noventa e nove ovelhas machos e fêmeas dos Estados de São Paulo (SP) e Rio de Janeiro (RJ) foram estudadas. Após o exame físico, amostras de lavagem traqueobrônquica foram obtidas. A presença de bactérias aeróbias e *Mycoplasma* spp. foram estudados, assim como a associação entre os microrganismos e estado clínico e sinais clínicos de doença respiratória em ovinos. As principais manifestações clínicas observadas foram: taquipneia (75%), alta temperatura retal (09,4%), secreção nasal mucopurulenta/purulenta (21,9%), tosse (25%), dispneia (31,2%), sons pulmonares alterados na ausculta (87,5%) e na percussão torácica (28,1%) em ovelhas pneumônicas. Bactérias gram-negativas não fermentadoras e *Bacillus* sp. foram as bactérias mais isoladas. Microrganismos da classe Mollicutes foram detectados molecularmente (PCR) em 33,3% dos ovinos. Além disso, descreve-se pela primeira vez no estado de São Paulo, Brasil, a detecção do

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M. mycoides subsp. *capri* na espécie ovina utilizando a reação de polimerase em cadeia.

TERMOS DE INDEXAÇÃO: Bactérias, trato respiratório inferior, ovinos, ovelhas, clínica, doença respiratória ovina, *Mycoplasma* spp., pneumonia, bacterioses.

INTRODUCTION

Sheep respiratory disease is one of the most important diseases in sheep flocks (Marcondes et al. 2011, Radostits et al. 2017) due to its economic losses. In New Zealand, pneumonia in lambs cost NZ\$28.1 million in 2008 (Goodwin-Ray et al. 2008). From 1996 to 2010, 10% of diagnosed diseases in lambs in the “Universidade Federal do Mato Grosso do Sul” were pneumonic related (Almeida et al. 2013). The incidence of pneumonia in sheep and other respiratory diseases in Argentina was 57.9% (Suárez & Buseti 2009). In Tanzania, 31.4% of lungs had pneumonic lesions in slaughter (Mellau et al. 2010). Recently, McRae et al. (2016) detected pneumonic lesions in 28% of lambs at slaughter in New Zealand.

Important risk factors related to respiratory disease of ruminants are the environmental high temperature and humidity, increased animal density, stress, dust, poor ventilation, and parasites (Goodwin-Ray et al. 2008, Taylor et al. 2010, Scott 2011). Some viruses are considered primary pathogens, changing the epithelial cells, and interfering in the immune response (Bosch et al. 2013), leading to secondary bacterial infections. According to Sharp & Nettleton (2007) acute respiratory disease is not caused by virus infections alone.

Mannheimia (*M.*) *haemolytica* and *Pasteurella* (*P.*) *multocida* are important respiratory pathogens in sheep flocks (Radostits et al. 2017). Both bacteria belong to family Pasteurellaceae, order Pasteurellales (Griffin 2010). These bacteria are part of the respiratory tract microbiota in sheep (Viana et al. 2007) and as stated before, immunosuppression and viral infections predispose to respiratory disease caused by both species (Bosch et al. 2013, Viana et al. 2007). Almost 10% of pneumonic sheep were positive for *P. multocida* in lungs in Nigeria (Odugbo et al. 2006). Ten sheep died during an outbreak of *M. haemolytica* associated pneumonia in the State of Minas Gerais in 2009 (Araujo et al. 2009).

Mycoplasma spp. belongs to class Mollicutes, order Mycoplasmatales and family Mycoplasmataceae (Razin & Hayflick 2010). They are the smallest organisms with self-replication (Razin & Hayflick 2010) and some are detected in healthy and diseased ruminants (Ayling et al. 2004, Kumar et al. 2011, Oliveira et al. 2016, Tortorelli et al. 2017, Gaeta et al. 2018). *M. mycoides* subsp. *capri* (Srivastava et al. 2000, Al-Momami et al. 2011, Kumar et al. 2013), *M. agalactiae* (Kumar et al. 2014), *M. capricolum* subsp. *capricolum* (Fischer et al. 2012), *M. capripneumoniae* (Igbal et al. 2019) and *M. ovipneumoniae* (George & Carmichael 1975, Ayling et al. 2004, Dassanayake et al. 2010) are described as important respiratory mycoplasmas for small ruminants.

Sheep with respiratory disease often show depression, inappetence, high rectal temperature, dyspnea, coughing, mucopurulent or purulent nasal discharge and increased cardiac and respiratory rates (Scott 2011). Only a few studies aimed detect the bacterial etiology of pneumonic sheep in Brazil (Coutinho et al. 2009, Marcondes et al. 2011). Therefore, herein it was detected the bacterial etiology of respiratory

disease and their relationship to clinical signs of sheep respiratory disease.

MATERIALS AND METHODS

All procedures were carried out in agreement with the guidelines of the Committee of Ethics on Animal Use (Protocol no. 3498060716).

In this cross-sectional research, 99 male and female sheep from 12 flocks from the States of São Paulo (SP=6) and Rio de Janeiro (RJ=6) were enrolled in this study, according to a non-probabilistic sampling for convenience.

Animals were classified as healthy and unhealthy (showing clinical signs of respiratory disease) after physical exam (Benesi et al. 2013, Gaeta et al. 2018). Sheep were considered unhealthy if show at least two of the following parameters: respiratory rate more than 30 respiratory movements per minute, coughing, rectal temperature higher than 40°C and abnormal sound on thoracic auscultation.

Tracheobronchial wash samples were obtained from both health and pneumonic sheep (Anton & Mayayo 2007). Briefly, trichotomy and antisepsis using 70% alcohol, chlorhexidine and iodopovidone were performed in the third part of the trachea. An Intracath® (BD, USA) was introduced by tracheocentesis and 20 to 50mL of sterile saline 0.9% was instilled, recovering up to 5mL. An aliquot (500µL) was added to Stuart transport media and stored at 4°C until the isolation of bacteria. Another aliquot was added to transport media for *Mycoplasma* spp. containing glycerol and stored in liquid nitrogen.

Tracheal suspensions were inoculated in Brain Heart Infusion broth and the cultures in agar plates were performed by plating 10µL of these samples in MacConkey and Blood agar (5%). The cultures were incubated for 24 to 72 hours at 37°C in aerobic atmosphere. The obtained colonies were noted for their morphology, Gram stained and screened for biochemical tests (*Staphylococcus* sp.: catalase test, sensitivity to bacitracin and furazolidone and oxidase test; *Streptococcus* sp.: catalase test, sensitivity to bacitracin, growth at 65% NaCl, bile-esculin test and growth at 45°C; *Bacillus* sp.: catalase test; *Klebsiella* sp. and non-fermenting bacteria: triple sugar iron growth, production of indole, urease and fenilalanine desaminase, oxidation-fermentation test, motility test, lysine descarboxylation test, use of citrate and malonate, lactose and glucose fermentation, nitrate reduction, Voges-Proskauer test and Methyl-red test) (Murray et al. 1999).

Mycoplasma spp. isolation was performed using solid and liquid SP-4 media (Tully 1995). Agar plates and broths were incubated in aerobiosis at 37°C for 15 days. *Mycoplasma* spp. were screened for producing “fried-egg”-like colonies and differentiated for glucose fermentation and/or arginine hydrolysis without turbiding the broths. Polymerase chain reaction (PCR) was performed to confirm the DNA of Mollicutes in samples (Van Kuppeveld et al. 1992). Then, the PCR positive samples were tested again for *M. bovis* and *M. agalactiae* (Chávez González et al. 1995) and *M. mycoides* subsp. *capri* (Monnerat et al. 1999).

The association between health status and clinical signs and microbiologic findings were calculated using Chi-square test or Fisher’s Exact test. Variables with a *P*-value <0,05 were considered significant. All calculations were performed using Software Statistical Package for Social Sciences 16.0 (SPSS, IBM).

RESULTS

After clinical examination, the sheep were classified as healthy (67.7%, 67/99) and pneumonic (32.32%, 32/99). Clinical signs such as tachypnea, high rectal temperature, mucopurulent/purulent nasal discharge, coughing, dyspnea, abnormal pulmonary sounds on auscultation and thoracic percussion were increased in pneumonic sheep compared to health sheep (Table 1).

Forty-five bacterial isolates were obtained. *Bacillus* sp., non-fermentative gram-negative bacteria, *Streptococcus* sp. and *Staphylococcus* sp. were the main species. *P. multocida* and *M. haemolytica* were not isolated (Table 2). Fried-egg-like

colonies were observed in 23.3% (17/73) of samples. The PCR methodology with generic primers for *Mollicutes* spp. confirmed all isolates of *Mollicutes*. In addition, PCR was also performed directly in tracheobronchial wash samples, and the DNA of *Mollicutes* spp. was detected in 45.2% (33/73) of samples (38% in pneumonic sheep and 49% in healthy sheep). *M. mycoides* subsp. *capri* (01/33) was detected by PCR. The used specific primers in this study did not allow detect other *Mollicute* species. No association between species detected and health status were detected.

Regarding to clinical manifestation, only *Bacillus* sp. was associated to respiratory rate ($P=0.005$) and submassive/massive sound on thoracic percussion ($P=0.024$).

Table 1. Clinical signs detected in healthy and pneumonic sheep from the States of São Paulo and Rio de Janeiro, Brazil

Clinical signs	Healthy % (N/T)	Pneumonic % (N/T)	P-value
Body Condition Score			
<2	73.1 (49/67)	42.9 (15/32)	0,037*
>2	26.9 (18/67)	53.1 (17/32)	
Heart rate (bpm)			
<120	67.7 (46/67)	50.0 (16/32)	0.073
>120	31.3 (21/67)	50.0 (16/32)	
Respiratory rate (breaths/min)			
<30	59.7 (70/67)	25.0 (08/32)	0.001*
>30	40.3 (27/67)	75.0 (24/32)	
Rectal temperature (°C)			
38.5 - 40.0	97.0 (65/67)	90.6 (29/32)	0,011*
>40.0	03.0 (02/67)	09.4 (03/32)	
Nasal discharge			
Normal	98.5 (66/67)	79.1 (25/32)	0,001*
Altered ^a	01.5 (01/67)	21.9 (07/32)	
Cough			
Absent	97.0 (65/67)	75.0 (24/32)	0,001*
Present	03.0 (02/67)	25.0 (08/32)	
Dyspnea			
Absent	94.0 (63/67)	68.8 (22/32)	0,001*
Present	06.0 (04/67)	31.2 (10/32)	
Breathing Pattern			
Costoabdominal	98.5 (66/67)	98.6 (29/32)	0,098
Costal/Abdominal	01.5 (01/67)	09.4 (03/32)	
Laringotracheal sound			
Normal	98.5 (66/67)	84.4 (27/32)	0,013*
Loud	01.5 (01/67)	15.6 (05/32)	
Percussion			
Clear	94.0 (63/67)	71.9 (23/32)	0,002*
Submassive/massive	06.0 (04/67)	28.1 (09/32)	
Auscultation			
Normal	86.6 (58/67)	12.5 (04/32)	<0,001*
Altered	13.4 (09/67)	87.5 (28/32)	
Fine Crackles	06.0 (04/67)	53.1 (17/32)	<0,001*
Thick Crackles	01.5 (01/67)	09.4 (03/32)	
Snoring	03.0 (03/67)	18.8 (06/32)	0,005*
Whistle	03.0 (03/67)	03.1 (01/32)	1,000

^a Mucopurulent/purulent; * means $P<0.05$ (Chi-Square Test or Fisher's Exact Test); N = number of observations, T = total of sheep.

Table 2. Aerobic bacteria detected in tracheobronchial wash samples of sheep in relation to respiratory health status

Bacteria	Healthy % (N/T)	Pneumonic % (N/T)	Total % (N/T)	P-value
<i>Bacillus</i> sp.	27.6 (08/29)	43.7 (07/16)	33.3 (15/45)	0.131
<i>Streptococcus</i> sp.	24.1 (07/29)	-	15.5 (07/45)	0.096
<i>Staphylococcus</i> sp.	20.7 (06/29)	25.0 (04/16)	22.2 (10/45)	0.484
NFGN	24.1 (07/29)	18.7 (03/16)	22.2 (10/45)	0.980
<i>Klebsiella oxytoca</i>	-	06.2 (01/16)	02.2 (01/45)	0.303
<i>Klebsiella pneumoniae</i>	-	06.2 (01/16)	02.2 (01/45)	0.303

N = number of isolates of the specific bacterium, T = total number of isolates.

DISCUSSION

The present study helps to better understand the main bacteria involved in the respiratory disease of Brazilian sheep from the States of São Paulo and Rio de Janeiro, Brazil. Bronchopneumonia was detected in 32.3% of sheep as detected in other studies in Brazil (Viana et al. 2007) and Ethiopia (Garedew et al. 2010).

Respiratory rate, rectal temperature, nasal discharge, cough, dyspnea, percussion and altered sounds in pulmonary auscultation were increased in pneumonic sheep ($P < 0.05$). These findings confirm the importance of the observed clinical signs of sheep to determine the respiratory health during physical examination (Viana et al. 2007, Legesse et al. 2018).

Opportunistic agents may have a controversy importance due their detection in healthy hosts. *Bacillus* sp. was the most isolated bacteria, especially in pneumonic sheep. This microbial genera was also detected in other studies with the same propose (Asaye et al. 2015, Megra et al. 2006, Gebremeskel et al. 2017, Yegoraw et al. 2017, Yimer & Asseged 2007). In fact, *Bacillus* sp. is an opportunistic infectious agent that belongs to the ovine respiratory tract microbiota (Rajivkumar & Ghaar 2000, Garedew et al. 2010). The presence of non-anthraxis *Bacillus* microorganism in clinical samples was considered a paradigm for a long time. As mentioned in literature, many infectious agents were previously considered not be pathogenic and became be accepted as pathogenic (Farrar Jr. 1963) as happened with *Bacillus* sp. considered a non-contaminant in clinical samples (Shimoyama et al. 2017). Otherwise *Bacillus cereus* is known to be responsible for nosocomial pneumonia in human, and in immunocompromised or immunocompetent human patients (Gray et al. 1999, Miyata et al. 2013, Shimoyama et al. 2017). *Bacillus* sp. has been isolated from lung tissue (Elshafee 2003) and tracheobronchial flushing samples of cattle (Oliveira et al. 2016, Gaeta et al. 2018). The present study stated the association of clinical signs of sheep, the respiratory disease and the isolation of *Bacillus* sp. (high respiratory rate and the presence of abnormal sound on thoracic percussion).

Klebsiella oxytoca and *K. pneumoniae* are virulent bacteria that are often associated with pneumonia in sheep (Ajuwape & Aregbesola 2002, Patel et al. 2017). Herein, both species were detected, as well was in other studies in Ethiopia et al. (2010) and Iraqi (Al-Sultan 1995).

Mycoplasmas are frequently related to respiratory disease in sheep, and are responsible for important economic losses (Kumar et al. 2012). Mollicutes were detected by PCR in 45.2% of studied sheep tracheal samples. Al-Momani et al. (2006) obtained similar results in Jordan (35% samples). In addition, we detected Mollicutes in 38% of pneumonic

and 49% in healthy sheep. It is well established that Mollicutes are part of the respiratory tract microbiota of ruminants, and after a stress, they may become pathogenic. These findings are also in agreement with other studies Swedish flocks (Tauni 2017), and India (Kumar et al. 2012). Mollicutes have also been detected in the lower respiratory tract of calves (Oliveira et al. 2016, Gaeta et al. 2018) in Brazil. *M. mycoides* subsp. *capri* was detected by PCR in present study in a healthy sheep. Opportunistic bacteria are easily spread into the flocks, particularly in animals in close proximity. As stated before, any situation that compromises the immune system favors the disease development. During a respiratory outbreak by *M. mycoides* subsp. *capri*, Hernandez et al. (2006) described a 60% mortality in a Mexican goat flock. Animals showed abundant nasal discharge, fever, dyspnea, prostration, ear drop, and decrease in milk production. Depending on the number of affected animals, economic losses might be severe. Finally, most of Mollicutes detected by PCR in studied samples was not specifically identified. This questioned the role of other species of Mollicutes in respiratory disease of sheep, such as *M. bovis* (Kumar et al. 2012), *Acholeplasma* sp., and *M. arginine* (DaMassa et al. 1992).

CONCLUSIONS

In the present study, *Bacillus* sp. was the main bacteria detected in both healthy and pneumonic sheep. Important pathogens such as *Klebsiella oxytoca* and *K. pneumoniae* were both isolated in pneumonic sheep, confirming their importance in the etiology of the disease in the southeastern region of Brazil.

Mollicutes is part of the ruminant's microbiota, but they are also important to ovine pneumonia.

This seems to be the first study that detected *Mycoplasma mycoides* subsp. *capri* in the State of São Paulo.

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***Herbaspirillum seropedicae* as a degrading bacterium of monofluoroacetate: effects of its inoculation in goats by ingesting *Amorimia septentrionalis* and the concentrations of this compound in plants sprayed with the bacterium¹**

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ABSTRACT- Pessoa D.A.N., Lopes J.R.G., Souza E.M., Campos E.M., Medeiros R.M.T., Cook D., Lee S.T. & Riet-Correa F. 2019. *Herbaspirillum seropedicae* as a degrading bacterium of monofluoroacetate: effects of its inoculation in goats by ingesting *Amorimia septentrionalis* and the concentrations of this compound in plants sprayed with the bacterium. *Pesquisa Veterinária Brasileira* 39(10):802-806. Unidade Acadêmica de Medicina Veterinária, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária s/n, Bairro Santa Cecília, Patos, PB 58700-970, Brazil. E-mail: danipessoavet14@gmail.com

Herbaspirillum seropedicae is a nitrogen-fixing bacterium capable of using toxic compounds as a source of carbon. Bacteria with this capacity can be used to make animals resistant to plant poisoning containing monofluoroacetate (MFA), such as *Amorimia septentrionalis*. The aim of this study was to evaluate if *H. seropedicae* is efficient in the degradation of MFA present in *A. septentrionalis* and if the inoculation of this bacterium in goats confers protection to *A. septentrionalis* intoxication. Two experiments were performed: in the first experiment 12 goats were divided into 2 groups. Goats in Group 1 were orally administered a solution containing the *H. seropedicae* bacterium for 10 days. From day 10 onwards, they received a daily dose of 5g/kg of *A. septentrionalis* with the bacteria until clinical signs of intoxication were observed. Group 2 goats received only the plant at the same dose, also until the observation of clinical signs of intoxication. The amount of MFA found in *A. septentrionalis* used in the experiment with goats was $1.6 \pm 0.058 \mu\text{g}/\text{mg}$. The total plant dose ingested by all goats in Group 1 was $80.83 \pm 12.81 \text{ g}/\text{kg}$ ($129.33 \pm 20.50 \text{ mg}/\text{kg}$ MFA), which were significantly greater ($p < 0.05$) than those of Group 2 goats ($39.16 \pm 19.08 \text{ g}/\text{kg}$ plant and $62.66 \pm 30.53 \text{ mg}/\text{kg}$ MFA). Group 1 goats took an average of 16.16 ± 2.56 days to develop clinical signs of intoxication, significantly longer ($p = 0.0012$) than Group 2 goats (7.83 ± 3.81 days). Two Group 2 goats died on the same day that they developed clinical signs of intoxication. At necropsy of these two animals, no significant changes were observed. In the second experiment, samples of *A. septentrionalis* were sprayed with a solution containing *H. seropedicae*. Before and eight days after spraying, the samples were pressed and dried for quantitation of MFA. The amount of MFA present in samples of *A. septentrionalis* 8 days after spraying with *H. seropedicae* was significantly lower ($p = 0.017$) than that found prior to spraying. It can be concluded

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that administration of *H. seropedicae* in goats is capable of causing greater resistance to *A. septentrionalis* intoxication, and spraying the plant with this bacterium significantly reduces the amount of MFA in the plant.

INDEX TERMS: *Herbaspirillum seropedicae*, degrading bacterium, monofluoroacetate, goats, *Amorimia septentrionalis*, plants sprayed, poisoning, toxic plants.

RESUMO.- [*Herbaspirillum seropedicae* como bactéria degradadora de monofluoroacetato de sódio: efeitos de sua inoculação em caprinos ingerindo *Amorimia septentrionalis* e nas concentrações deste composto em plantas pulverizadas com a bactéria.]

Herbaspirillum seropedicae é uma bactéria fixadora de nitrogênio, capaz de utilizar compostos tóxicos como fonte de carbono. Bactérias com essa capacidade podem ser utilizadas para tornar os animais resistentes à intoxicação por plantas que contêm monofluoroacetato (MFA), como *Amorimia septentrionalis*. O objetivo do presente estudo é avaliar se *H. seropedicae* é eficiente na degradação do MFA presente em *A. septentrionalis* e se a inoculação dessa bactéria, em caprinos, confere proteção à intoxicação por *A. septentrionalis*. Foram realizados dois experimentos: no primeiro experimento foram utilizados 12 caprinos, divididos em dois grupos. Os caprinos do Grupo 1 receberam diariamente, oralmente, uma solução contendo a bactéria *H. seropedicae* durante 10 dias. A partir do décimo dia passaram a receber, diariamente, além da solução com a bactéria 5g/kg de *A. septentrionalis* até a observação de sinal clínico de intoxicação. Os caprinos do Grupo 2 receberam apenas a planta na mesma dose, também até que a observação de sinais clínicos de intoxicação. A quantidade de MFA encontrada em *A. septentrionalis* utilizada no experimento com caprinos foi de $1,6 \pm 0,058 \mu\text{g/mg}$ de planta em média. A dose total de planta ingerida por todos os caprinos do Grupo 1 foi de $80,83 \pm 12,81 \text{g/kg}$ ($129,33 \pm 20,50 \text{mg/kg}$ de MFA), valores significativamente maiores ($p < 0,05$) do que os dos caprinos do Grupo 2 ($39,16 \pm 19,08 \text{g/kg}$ de planta e $62,66 \pm 30,53 \text{mg/Kg}$ de MFA). Os caprinos do Grupo 1 demoraram em média $16,16 \pm 2,56$ dias para desenvolver sinais clínicos da intoxicação, período significativamente maior ($p = 0,0012$) que os caprinos do Grupo 2 ($7,83 \pm 3,81$ dias). Dois caprinos do Grupo 2 morreram no mesmo dia que desenvolveram sinais clínicos da intoxicação. Na necropsia desses dois animais não foram observadas alterações significativas. No segundo experimento, amostras de *A. septentrionalis* foram pulverizadas com uma solução contendo a bactéria *H. seropedicae*. Antes e oito dias após a pulverização, as amostras foram prensadas e secas para posterior quantificação do MFA. A quantidade de MFA presente nas amostras de *A. septentrionalis* oito dias após a pulverização com *H. seropedicae* foi significativamente menor ($p = 0,017$) do que a encontrada antes da pulverização. Pode-se concluir que a administração de *H. seropedicae* em caprinos é capaz de causar uma maior resistência à intoxicação por *A. septentrionalis*, e a pulverização da planta com esta bactéria reduz significativamente a quantidade de MFA na planta.

TERMOS DE INDEXAÇÃO: *Herbaspirillum seropedicae*, bactéria degradadora, monofluoroacetato de sódio, caprinos, *Amorimia septentrionalis*, plantas tóxicas.

INTRODUCTION

Ruminant poisoning by toxic plants causes significant losses in the Brazilian herd. It is estimated that 52,675 to 63,292 goats die each year in Brazil from plant poisoning. In cattle this number may exceed 1 million head of cattle, and half of these deaths

are due to poisoning by plants containing monofluoroacetate (MFA) (Pessoa et al. 2013).

In Brazil, 22 species of plants that cause sudden death associated with exercise in ruminants are known, among them *Amorimia septentrionalis*, previously identified as *Amorimia (Mascagnia) rigida*, and popularly known as tinguí. *A. septentrionalis* found in northeastern Brazil, mainly in the States of Paraíba, Pernambuco and Ceará (Duarte et al. 2013, Nascimento et al. 2018) and is responsible for outbreaks of poisoning in goats and sheep in Paraíba (Vasconcelos et al. 2008) and in cattle in both Paraíba and Pernambuco (Albuquerque et al. 2014).

Control and prevention of this type of poisoning by eliminating the plant or by avoiding its consumption by animals is not always effective (Barbosa et al. 2003). New alternatives are suggested to make animals resistant to poisoning, including: repeated administration, at alternate periods, of non-toxic doses of the plant (Duarte et al. 2014); the transfer of ruminal fluid from ruminants inoculated with MFA-degrading bacteria that became resistant to intoxication to susceptible ruminants (Silva et al. 2015); the administration of MFA-degrading bacteria isolated from the rumen of goats, soil and plants containing MFA (Pessoa et al. 2015, Silva et al. 2016, Pessoa et al. 2018); and the use of lithium chloride as a conditioned aversion technique (Brito et al. 2016). Among these alternatives is the use of bacteria capable of degrading the MFA present in the plant. When animals are administered these bacteria daily and continuously, the animals develop different degrees of resistance to poisoning by plants containing MFA (Silva et al. 2016, Pessoa et al. 2018).

Herbaspirillum seropedicae is a gram-negative, rod-shaped, aerobic, positive-motility bacterium belonging to the β subdivision of proteobacteria that uses organic and inorganic acids as a carbon source (Schmid et al. 2006). It is also considered diazotrophic and endophytic facultative, i.e. it is able to fix nitrogen under microaerophilia conditions and colonize the leaves, stems, and roots of grasses of great economic interest such as corn, wheat, sorghum, rice and sugarcane (Roncato Maccari et al. 2003, Baldani & Baldani 2004, Rodrigues et al. 2006). It has flexible metabolism, capable of degrading various organic and inorganic compounds, most often through the production of enzymes, such as dehalogenases, which are able to catalyze the breakdown of the carbon-halogen bond in halogenated compounds, such as monofluoroacetate, rendering it non-toxic (Fetzner & Lingens 1994, Pedrosa et al. 2011). Due to this versatility in the degradation capacity of toxic compounds, the objective of the present study was to evaluate the use of *H. seropedicae* as an MFA-degrading bacterium, either by oral administration in goats exposed to *Amorimia septentrionalis* poisoning or by field spraying of *A. septentrionalis* specimens with the bacterium. For this, two experiments were performed, one with the administration of bacteria in goats, and the other by spraying the plant with the bacteria.

MATERIALS AND METHODS

Experiment 1. *Amorimia septentrionalis* used in the study was collected in the municipality of Teixeira (7°12.24' S 37°15.11' W; elevation 749m), Paraíba. A plant *exsiccata* was deposited at the Herbarium of the "Universidade Federal de Campina Grande" (UFCG), at the Patos Campus, Paraíba, Brazil (registration No. 6701). To determine the amount of monofluoroacetate present in the plant, a five plant samples, each with 10 leaves, were collected, pressed and dried, and then sent to the Poisonous Plant Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, USA for analysis by high performance liquid chromatography mass spectrometry (Lee et al. 2012).

The degrading bacterium of MFA *Herbaspirillum seropedicae*, used in this study, is part of the SmR1 strain. It was obtained in partnership with the Department of Biochemistry of the UFCG.

To obtain the solution used, *H. seropedicae* was grown in NFBHP lactate agar medium supplemented with NH_4Cl for 48 hours at 30°C (Machado et al. 1991) in a bacteriological greenhouse incubator. After it was diluted in 60mL NaCl at 0°C. 9% sterile until reaching Mac Farland scale grade 1 (Gregg et al. 1998). After testing by the plate colony counting method, it was determined that at this degree of turbidity each 1mL of the solution contained approximately 8.6×10^7 bacterial colony forming units.

Twelve crossbred goats were used, ranging in age from 1 to 4 years, and weighing 25 to 45kg, coming from free areas of plants containing MFA. These were divided into two groups, each with six animals. Group 1 goats were daily administered an oral dose of a 60mL of the solution containing *H. seropedicae* for 10 days (Silva et al. 2016). From the tenth day and until clinical signs of intoxication were observed, 5g/kg of *A. septentrionalis* was associated with the solution with the bacteria. Group 2 goats received only *A. septentrionalis* at the same dose, also until clinical signs of intoxication were observed.

The plant was offered to goats on a voluntary basis and those that did not eat received it by administering small amounts directly into their mouths. After administration, all animals were stimulated to move for 10 minutes. Daily, before and after administration of the plant, rectal temperature, heart rate, respiratory rate and ruminal movements of all goats were measured and the presence of clinical signs of intoxication were observed. Animals that died were necropsied and organ fragments of the abdominal and thoracic cavities and central nervous system were collected, fixed in 10% formaldehyde, processed according to routine methods, stained with hematoxylin-eosin (HE), and subsequently evaluated histopathologically.

For statistical analysis, the variables were: day of observation of clinical signs of intoxication, amount of plant ingested by each goat, and amount of MFA ingested per kg of body weight. These values were submitted to the Shapiro-Wilk normality test and then expressed as means and standard deviations. The comparison between the values observed in the groups was made by Student's t-test for independent samples, both for the day of observation of clinical signs of intoxication and for the amount of plant ingested and MFA ingested (Zar 1999). The program used to perform the analyses was BioEstat 5.3, and the significance level adopted was 5%.

Experiment 2. For the spray test, *H. seropedicae* was diluted in 60ml of 0.9% sterile NaCl until it reached Mac Farland grade 1 climbing. Thereafter it was mixed in one liter of NFBHP lactate broth supplemented with NH_4Cl and was then incubated in a bacteriological oven for 48 hours at 30°C (Machado et al. 1991). Eight liters of the medium solution with the bacteria were used for the study. After

the incubation period, they were placed in sterile hand sprayers for subsequent spraying of the plants.

The area chosen for spraying was Sítio Lopes, a property in the city of Teixeira (7°12.24' S 37°15.11' W; elevation 749m), Paraíba, Brazil, which had a significant amount of *A. septentrionalis*. Twenty-five plant specimens were sprayed over an area of 100 m², where approximately every plant received 320 ml of solution. Spraying was carried out during the dry season, early in the morning, once. After eight days the plants were collected, pressed and dried. Five samples, each with 10 leaves, were sent to the Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, USA, for the quantitation of MFA (Lee et al. 2012).

For statistical analysis, the MFA concentrations before and after spraying were subjected to the Shapiro-Wilk normality test and then expressed as means and standard deviations. The comparison between means before and after spraying was done by Student's t-test for related samples (Zar 1999). The program used to perform the analyses was BioEstat 5.3, and the significance level adopted was 5%.

RESULTS

Experiment 1

All goats showed clinical signs of intoxication, which consisted of tachypnea, tachycardia, arrhythmia, bleating, tremors, motor incoordination, reluctance to movement, anorexia, apathy, fall, distended neck, and engorged jugular vein with positive venous pulse (Fig.1). The period for the development of clinical signs of poisoning in Group 1 goats that received the bacterium was 16.16 ± 2.56 days, significantly longer ($p=0.0012$) than Group 2 goats, which received was 7.83 ± 3.81 days (Table 1).

Considering that the amount of MFA found in *Amorimia septentrionalis* was $1.6 \pm 0.58 \mu\text{g}/\text{mg}$, the 5g/kg dose of plant supplied to the animals corresponds on average to 8 mg MFA per kg of body weight. The total plant dose ingested by Group 1 goats was $80.83 \pm 12.81 \text{g}/\text{kg}$, therefore goats ingested $129.33 \pm 20.50 \text{mg}/\text{kg}$ MFA in total, while Group 2 goats ingested $39.16 \pm 19.08 \text{g}/\text{kg}$ plant and $62.66 \pm 30.53 \text{mg}/\text{kg}$ MFA (Table 1).



Fig.1. Control goat with severe depression and sternal decubitus after ingestion of *Amorimia septentrionalis*.

Table 1. Period of observation of clinical signs in *Amorimia septentrionalis* poisoning, amount of plant ingested and amount of MFA ingested until the onset of clinical signs in Group 1 goats, which ingested *Herbaspirillum seropedicae* and *A. septentrionalis*, and Group 2 goats, which ingested only the plant

Group/Goats	Day of observation of clinical signs of intoxication*	Dose of ingested plant (g/kg)*	MFA intake dose (mg/kg)*
Group 1			
1	18	90	144
2	20	100	160
3	14	70	112
4	14	70	112
5	17	85	136
6	14	70	112
Average ± standard deviation	16.16 ± 2.56 ^a	80.83 ± 12.81 ^a	129.33 ± 20.50 ^a
Group 2			
7	5	25	40
8	6	30	48
9**	11	55	88
10**	3	15	24
11	13	65	104
12	9	45	72
Average ± standard deviation	7.83 ± 3.81 ^b	39.16 ± 19.08 ^b	62.66 ± 30.53 ^b

* Student's t-test, ** animals 9 and 10 died on the same day showing clinical signs of intoxication; ^{a,b} different letters in the same column indicate statistical difference (p<0.05) between the groups.

Table 2. Amount of MFA measured before and eight days after spraying *Amorimia septentrionalis* specimens with *Herbaspirillum seropedicae* bacteria

Sample	Amount of MFA (µg/mg)	
	Before spraying	Eight days after spraying
A 1	0.61	0.23
A 2	0.85	0.27
A 3	1.2	0.32
A 4	1.4	0.19
A 5	2.0	0.22
Average ± standard deviation*	1.21 ± 0.53 ^a	0.24 ± 0.05 ^b

* Student's t-test for related samples; ^{a,b} different letters in different columns represent statistically significant difference (p<0.05).

Both the amount of plant and MFA ingested by Group 1 goats were significantly greater (p<0.05) than Group 2 goats.

Goats in both Groups (1 and 2) recovered on average three days after the end of plant ingestion. However, in two Group 2 goats, the clinical signs of poisoning evolved to death on the same day they were observed, and no significant changes were observed in the necropsy of these animals.

Experiment 2

The amount of MFA present in *A. septentrionalis* samples eight days after *Herbaspirillum seropedicae* spraying (0.24±0.05µg/mg) was significantly lower (p=0.017) than before spraying (1.21±0.53µg/mg) (Table 2).

DISCUSSION

The results of the present study show that goats that ingested *Herbaspirillum seropedicae* took longer to develop clinical signs of *Amorimia septentrionalis* poisoning, containing 0.16% MFA, than goats that did not receive the bacteria.

In addition, in the goats that received the bacteria, no deaths were recorded, while in the group that did not receive the bacteria two goats died. This suggests that the bacteria likely degraded the MFA contained in the plant. These results are similar to those obtained with other MFA-degrading bacteria (Pessoa et al. 2015, 2018, Silva et al. 2016).

Silva et al. (2016) presented more satisfactory results than those of this study since resistance remained during the 30 days of administration of *Ralstonia* sp. and *Burkholderia* sp. without goats showing any clinical signs of intoxication. In the study by Silva et al. (2016) the authors do not mention the MFA concentration of the plant used. However, the amount of MFA detected in *A. septentrionalis* samples used in experiment 1 (0.16%) was higher than that found in previous studies: 0.002% (Lee et al. 2012), 0.0021% (Albuquerque et al. 2014) and 0.00074% (Lopes et al. 2019). Marked variations in MFA content in *A. septentrionalis* are also reported by Lee et al. (2012). Lee et al. (2012) reported variations in MFA content in *A. septentrionalis*. It is likely that *H. seropedicae* does not survive or multiply in the rumen to effectively hydrolyze significant amounts of MFA as found in previously reported studies.

The bacteria *Ralstonia* sp., *Burkholderia* sp., *Paenibacillus* sp. and *Cupriavidus* sp. induce resistance to intoxication by *A. septentrionalis*; however, this resistance ends after a period of 55 days after the end of bacterial ingestion (Pessoa et al. 2018). In the case of *H. seropedicae*, which is an endophytic bacterium, it was also tested for colonization of the plant by degrading MFA. The results show a significant reduction in MFA concentrations in plants that were treated with *H. seropedicae*, suggesting that *H. seropedicae* may be able to colonize *A. septentrionalis*. Further experiments should be performed to prove these data and to verify the persistence of *H. seropedicae* in *A. septentrionalis* and probably in other plants containing MFA, mainly in *Palicourea* species, which contain higher concentrations of MFA and are more widespread in Brazil.

CONCLUSION

It was concluded that the inoculation of *Herbaspirillum seropedicae* in goats was able to cause greater resistance to *Amorimia septentrionalis* poisoning. Spraying samples of *A. septentrionalis* with *H. seropedicae* was able to significantly decrease the amount of MFA present in the plant.

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***Pseudomonas* spp. and other psychrotrophic microorganisms in inspected and non-inspected Brazilian Minas Frescal cheese: proteolytic, lipolytic and AprX production potential¹**

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ABSTRACT. Teider Junior P.I., Ribeiro Júnior J.C., Ossugui E.H., Tamanini R., Ribeiro J., Santos G.A., Alfieri A.A. & Beloti V. 2019. *Pseudomonas* spp. and other psychrotrophic microorganisms in inspected and non-inspected Brazilian Minas Frescal cheese: proteolytic, lipolytic and AprX production potential. *Pesquisa Veterinária Brasileira* 39(10):807-815. Instituto Nacional de Ciência e Tecnologia para a Cadeia Produtiva do Leite, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid PR-445 Km 380, Cx. Postal 10.011, Campus Universitário, Londrina, PR 86057-970, Brazil. E-mail: ribeirojuniorjc@gmail.com

The most consumed cheese in Brazil, Minas Frescal cheese (MFC) is highly susceptible to microbial contamination and clandestine production and commercialization can pose a risk to consumer health. The storage of this fresh product under refrigeration, although more appropriate, may favor the growth of spoilage psychrotrophic bacteria. The objective of this study was to quantify and compare *Pseudomonas* spp. and other psychrotrophic bacteria in inspected and non-inspected MFC samples, evaluate their lipolytic and proteolytic activities and their metalloprotease production potentials. Twenty MFC samples were evaluated: 10 inspected and 10 non-inspected. Counts of psychrotrophic bacteria and *Pseudomonas* spp., evaluation of the proteolytic and lipolytic potential of the isolates, and identification of potential producers of alkaline metalloprotease (AprX) were assessed. The mean total psychrotrophic counts were $1.07 (\pm 2.18) \times 10^9$ CFU/g in the inspected samples and $4.5 (\pm 5.86) \times 10^8$ CFU/g in the non-inspected, with no significant difference ($p=0.37$). The average score of *Pseudomonas* spp. was $6.86 (\pm 18.6) \times 10^5$ and $2.08 (\pm 3.65) \times 10^6$ CFU/g for the inspected and non-inspected MFC samples, respectively, with no significant difference ($p=0.1$). *Pseudomonas* spp. represented 0.06% and 0.004% of psychrotrophic bacteria found in inspected and non-inspected MFC samples, respectively. Collectively, 694 psychrotrophic strains and 47 *Pseudomonas* spp. were isolated, of which 59.9% and 68.1% were simultaneously proteolytic and lipolytic, respectively. Of the 470 psychrotrophs isolated from inspected and 224 from non-inspected cheese samples, 5.74% and 2.23% contained *aprX*, respectively, while 100 and 86.96% of the *Pseudomonas* spp. isolates in inspected and non-inspected cheese samples contained the gene. The production potential of AprX did not, however, determine the proteolytic activity on plates of these isolates under the conditions evaluated in this study. Of total, 65.63% of the psychrotrophs that contained *aprX* gene were confirmed as *Pseudomonas* spp., using genus-specific PCR. Phylogenetic analysis of the 16S rRNA gene of the other psychrotrophs that were potential producers of AprX identified them as *Serratia* spp. ($n=7$), *Raoultella ornithinolytica* ($n=1$), and *Acinetobacter schindleri* ($n=1$) in the inspected samples and *Psychrobacter sanguinis* ($n=1$) and *Leuconostoc mesenteroides* ($n=1$) in the non-inspected

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samples. The production conditions of Brazilian MFC of these samples, while meeting the legal determinations, are not sufficient to control *Pseudomonas* and other spoilage-related psychrotrophs. Thus, stricter hygienic measures are required during the formal production of this type of cheese.

INDEX TERMS: *Pseudomonas* spp., psychrotrophic, microorganisms, Minas Frescal cheese, cheese, AprX production, alkaline metalloprotease, proteolysis, lipolysis.

RESUMO.- [*Pseudomonas* spp. e outros micro-organismos psicrotróficos em queijos Minas Frescal inspecionados e não inspecionados: potencial proteolítico, lipolítico e produção de AprX.] O mais consumido no Brasil, o queijo Minas Frescal (QMF) é altamente suscetível à contaminação microbiana e a produção e comercialização clandestina podem representar um risco para a saúde do consumidor. O armazenamento deste produto fresco sob refrigeração, embora mais apropriado, pode favorecer a multiplicação de bactérias psicrotróficas deteriorantes. O objetivo deste estudo foi quantificar e comparar *Pseudomonas* spp. e outras bactérias psicrotróficas em amostras de QMF inspecionadas e não inspecionadas, avaliar o potencial lipolítico, proteolítico e de produção de metaloprotease alcalina. Vinte amostras de QMF foram avaliadas: 10 inspecionadas e 10 não inspecionadas. Foram avaliadas as contagens de bactérias psicrotróficas e *Pseudomonas* spp., o potencial proteolítico e lipolítico dos isolados e a identificação de potenciais produtores de metaloprotease alcalina (AprX). A média total das contagens de bactérias psicrotróficas foi de $1,07 (\pm 2,18) \times 10^9$ UFC/g nas amostras inspecionadas e $4,5 (\pm 5,86) \times 10^8$ UFC/g nas não inspecionadas, sem diferença significativa ($p=0,37$). A média de *Pseudomonas* spp. foi de $6,86 (\pm 18,6) \times 10^5$ e $2,08 (\pm 3,65) \times 10^6$ UFC/g para as amostras QMF inspecionadas e não-inspecionadas, respectivamente, sem diferença significativa ($p=0,1$). *Pseudomonas* spp. representaram 0,06% e 0,004% de bactérias psicrotróficas encontradas em amostras QMF inspecionadas e não-inspecionadas, respectivamente. Das amostras inspecionadas e não inspecionadas, foram isoladas 694 colônias psicrotróficas e 47 *Pseudomonas* spp., dos quais 59,9% e 68,1% foram simultaneamente proteolíticos e lipolíticos, respectivamente. Dos 470 isolados de psicrotróficos das amostras de queijo inspecionados e dos 224 isolados das não inspecionadas, 5,74% e 2,23% continham o gene *aprX*, respectivamente, enquanto 100 e 86,96% das *Pseudomonas* spp. isoladas em amostras de queijo inspecionadas e não inspecionadas continham o potencial de expressão de AprX. Esse potencial, no entanto, não determinou a atividade proteolítica em placas desses isolados nas condições avaliadas neste estudo. Do total, 65,63% dos psicrotróficos que continham o gene *aprX* foram confirmados como *Pseudomonas* spp., utilizando PCR gênero-específico. A análise filogenética do gene 16S rRNA dos outros psicrotróficos que foram produtores potenciais de AprX os identificou como *Serratia* spp. ($n=7$), *Raoultella ornithinolytica* ($n=1$) e *Acinetobacter schindleri* ($n=1$) nas amostras inspecionadas e *Psychrobacter sanguinis* ($n=1$) e *Leuconostoc mesenteroides* ($n=1$) nas amostras não inspecionadas. As condições de produção do QMF dessas amostras, atendendo às determinações legais, não são suficientes para controlar a *Pseudomonas* e outros psicrotróficos relacionados à deterioração. Assim, medidas higiênicas mais rígidas são necessárias durante a produção formal deste tipo de queijo.

TERMOS DE INDEXAÇÃO: *Pseudomonas* spp., micro-organismos, psicrotróficos, queijo, Minas Frescal, produção de AprX, metaloprotease alcalina, proteólise, lipólise.

INTRODUCTION

By definition, Brazilian Minas Frescal cheese (MFC) is a fresh product obtained by the enzymatic coagulation of milk with rennet and/or other suitable coagulating enzymes, with or without supplementation with specific lactic bacteria, powdered milk, cream, milk solids, sodium chloride, and calcium chloride (Brasil 1997). It is classified as a semi-fat (25 to 44.9% fat) cheese with very high moisture content (not less than 55.0%) (Brasil 2004). The production of this type of cheese in Brazil is carried out formally by dairies that follow the legal stipulations of the “Ministério da Agricultura, Pecuária e Abastecimento” (Brasil 1997) and mainly small milk producers, with the objective of adding value and increases the shelf life of milk through the preparation of derivatives.

The MFC is quite susceptible to microbial contamination when prepared using raw milk, or during or after processing (Carvalho et al. 2007). Moreover, it can be a source of food pathogens (Campos et al. 2017).

In addition to pathogenic microorganisms, proteolytic and/or lipolytic microorganisms, mainly psychrotrophs, may interfere with the quality of the MFC, since as it is a fresh product, refrigeration is necessary for its storage. When present in the raw material, psychrotrophic bacteria reduce the industrial yield, flavor, and aroma, being able to render the cheese improper for consumption (Murphy et al. 2016).

As the degradation of proteins results in non-acid metabolites, protease activity confers a bitter taste and putrid smell to the cheese. Undesirable effects are also influenced by the reduction of the integrity of milk proteins, deficient coagulation, and greater loss of casein fragments in the serum, requiring 20-30 percent more milk per kilo of cheese (Samaržija et al. 2012).

Alkaline metalloprotease (AprX), which is heat-resistant, is considered the main microbial protease, and is encoded by *aprX*. This gene is found in various proteolytic bacteria, such as *Pseudomonas* spp. and *Serratia* spp. (Dufour et al. 2008, Marchand et al. 2009, Bagliniere et al. 2013). This enzyme is of great significance to dairy industry, because it leads to the deterioration of casein, which causes significant alterations of the physical and chemical quality and organoleptic properties of raw milk and its derivatives (Dufour et al. 2008).

Milk fat is equally compromised by microbial activity. Microbial lipases elevate heat resistance and promote a rancid flavor and aroma in dairy products. Microbial lipases and proteases remain active even after the elimination of the vegetative forms of micro-organisms by pasteurization (Oliveira et al. 2015, Murphy et al. 2016), as in case of Minas cheese.

Several studies have demonstrated the predominance of the genus *Pseudomonas* among dairy psychrotrophs (Ozturkoglu-Budak et al. 2016, Vithanage et al. 2016, Xin et al. 2017). Several species of *Pseudomonas* are responsible for the deterioration of other refrigerated foodstuffs (Samaržija et al. 2012, Oliveira et al. 2015), since they have the capacity to produce proteolytic and lipolytic enzymes at different temperatures, which reinforces their importance as spoilage agents of the dairy product chain (Scatamburlo et al. 2015, Ribeiro Júnior et al. 2018). Furthermore, because they have a high capacity to form biofilms, allowing them to proliferate in a wide variety of environments (Murphy et al. 2016).

Taking into account the technical problems that spoilage microorganisms cause in dairy products, the objective of this study was to quantify and compare *Pseudomonas* spp. and other psychrotrophic bacteria in inspected and non-inspected MFC samples, evaluate their lipolytic and proteolytic activities and their metalloprotease production potentials by identification of *aprX* gene.

MATERIALS AND METHODS

Sampling and preparation of cheese. Twenty samples of MFC marketed in the municipality of Londrina/PR, from May to June 2017, were evaluated. Ten samples of different brands were collected from supermarkets and were recorded by the Brazilian state or federal inspection system and were thus regarded as formal, inspected. These inspected samples had from 11 to 41 days of manufacture, with 22 days on average.

The remaining 10 MFC samples were collected from different fairs in the municipality and were marketed in a clandestine manner, and were therefore considered non-inspected. It was not possible to determine the period between the manufacture date and the analysis of these non-inspected samples, since the sellers were not necessarily the producers.

The MFC samples evaluated did not present any type of alteration of coloration (white, slightly yellowish), texture (soft) or smell (slightly acidic). They had varying amounts of white or yellowish serum, characteristic of MFC.

The samples were transported under refrigeration to the Laboratory of Inspection of Products of Animal Origin, which is a part of the "Instituto Nacional de Ciência e Tecnologia para a Cadeia Produtiva do Leite" (INCT-Cadeia do Leite) of the "Universidade Estadual de Londrina" (UEL), Paraná, Brazil, where they were immediately processed.

The external surfaces of packaging were sanitized with 70% alcohol. For the psychrotrophic bacterial count, a 25-g aliquot obtained aseptically from different fragments of the cheese sample was homogenized with 225 mL of 0.1% peptone saline in Stomacher blender for 180 seconds, obtaining a 10^{-1} dilution. From this dilution, serial decimal dilutions were performed with the same diluent.

For estimating the count of *Pseudomonas* spp., another 25-g aliquot of each sample was diluted in 225 mL buffered peptone water (Oxoid®, England) and homogenized in a Stomacher blender, according to ISO 11.059 (ISO 2009) recommendations.

Counting of microorganisms. For estimating the psychrotrophic bacterial count, 0.1 mL of the dilutions were grown in duplicate on the surface of Plate Count Agar (PCA) (Acumedia, Baltimore, USA) plates and incubated at 7°C for 10 days.

The count of *Pseudomonas* spp. was performed according to ISO 11059 (2009). A tenth of mL of the dilutions was spread on the surface of penicillin pimarcin agar (PPA) plates, prepared with

Pseudomonas agar base (Oxoid) supplemented with 100,000 IU/L of penicillin G potassium (Sigma Aldrich®, United States), and 0.01 g/L of piramicin (Coalhopar F-E-B Biotecnologia®, Brazil). The plates were incubated at 25°C for 48h. All colonies were subjected to tests for oxidase and glucose fermentation. Only the oxidase positive, non-glucose fermentative colonies were taken into account for the counts of *Pseudomonas* spp.

The counts were compared by the t-test using the Statistica v. 6.0 software (StatSoft, OK, USA).

Proteolytic and lipolytic potential of *Pseudomonas* spp. and others psychrotrophic bacteria. The colonies of *Pseudomonas* spp. and psychrotrophic bacteria were inoculated onto milk agar plates (Acumedia) supplemented with a solution of 10% reconstituted milk powder (Molico®, Nestlé, São Paulo, Brazil), and in tributyrin agar (HiMedia, Mumbai, India) supplemented with 1% tributyrin (HiMedia) to assess the proteolytic and lipolytic activity, respectively, according to the procedure stated by Hantsis-Zacharov & Halpern (2007). The plates were incubated under the same conditions recommended for the bacterial counts.

DNA extraction. The psychrotrophic colonies that showed spoiling potential on plates were grown in brain heart broth (Merck®, Germany) and incubated at 35°C for 48h, under the same incubation conditions as that for the colonies of *Pseudomonas* spp. grown in tryptone soy broth (Oxoid). An aliquot of 1 mL of each broth was used to extract DNA by the simple boiling method, according to the study by Ribeiro Júnior et al. (2016).

Molecular confirmation of *Pseudomonas* spp. The isolates of *Pseudomonas* spp. obtained in the counts and the other psychrotrophs were subjected to PCR amplification of a specific region in the 16S rRNA gene of the genus *Pseudomonas*, according to the amplification protocol described by Spilker et al. (2004), using the primers F-GS-PA (GACGGTGAGTAATGCCTA) and PA-GS-R (CACTGGTGTTCCTCTCTATA). PCR reactions were performed using 50 ng of template DNA, 10 nM of each dNTP, 1× buffer, 75 mmol/L of MgCl₂, 20 pmol/L of each primer, and 2.5 U of Platinum Taq DNA polymerase (Invitrogen, CA, USA), to yield a final reaction volume of 50 µL, recommended by Ribeiro Júnior et al. (2016). Samples which displayed 618-bp amplicons were considered as *Pseudomonas* spp.

Detection of *aprX*. PCR of *aprX* gene (*AprX* enzyme) was performed using the primers *apr I* (TAYGGBTTCAAYTCCAAYAC) and *apr II* (VGCGATSGAMACRTTRCC) and amplification conditions described by Bach et al. (2001). The reaction conditions were the same as those cited above (Ribeiro Júnior et al. 2016), with 194-bp amplicons being considered positive.

Amplification and sequencing of the 16S rRNA gene. The isolates that were positive for *aprX* and were not confirmed as *Pseudomonas* spp. by genus-specific reaction were subjected to the partial amplification of the 16S rRNA gene using the primers 27f (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'-GGYTACCTTGTTACGACTT-3') (Osborne et al. 2005). The amplification conditions were as follows: 1 cycle of initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 1 min, annealing at 58°C for 1 min, and primer extension at 72°C for 1 min; and a final extension cycle at 72°C for 10 min.

The PCR product from the 16S rRNA gene was then purified (PureLink™ Genomic DNA Purification Kit, Invitrogen) and quantified (Qubit® dsDNA HS Assay Kit, Invitrogen). DNA sequencing was performed by the Sanger method (ABI 3500 Genetic Analyzer, Applied Biosystems, Foster City, USA) in both directions. A representative sequence of each species found was selected for deposit in GenBank.

Phylogenetic analysis for species identification. The quality of the 16S rRNA sequences was evaluated by BioEdit v. 7.2.5 software

(Hall 1999) and the consensual sequences were generated by CAP 3 (Huang & Madan 1999). Preliminary identification at the genus level was performed by the BLAST tool of the National Center for Biotechnology Information (NCBI). Once the genera were identified, the sequences were individually aligned by Clustal W with the representative type sequences of all species of the genus available in the Ribosomal Database Project (RDP)⁴; the identification of the species was based on the genetic identity matrix calculated by the Tamura-Nei model in the MEGA software v. 7.0 (Kumar et al. 2016). The phylogenetic trees were elaborated in the same software, using the Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates.

RESULTS

The psychrotroph counts ranged from 3.5×10^7 - 6.85×10^9 UFC/g in the inspected cheese samples, with a mean of $1.07 (\pm 2.18) \times 10^9$ UFC/g. In the non-inspected cheese samples, psychrotroph counts ranged between 2.6×10^7 - 1.65×10^9 UFC/g, with a mean of $4.5 (\pm 5.86) \times 10^8$ CFU/g. No significant difference was observed between the psychrotroph counts in the inspected and non-inspected cheese samples ($p=0.37$).

The mean of *Pseudomonas* spp. counts was $6.86 (\pm 18.6) \times 10^5$ and $2.08 (\pm 3.65) \times 10^6$ CFU/g for formally and informally marketed cheese, respectively. There was no significant difference ($p=0.1$) between the counts of *Pseudomonas* spp. among the inspected and non-inspected cheeses, although the average of counts of *Pseudomonas* spp. in inspected cheese samples was only 32.9% of that in the non-inspected cheese samples.

⁴ Ribosomal Database Project (RDP), Center for Microbial Ecology, Michigan State University, Michigan, USA. Available at <<https://rdp.cme.msu.edu/hierarchy>>

From the plates used for the psychrotroph counts, 694 colonies were isolated: 470 from inspected cheese and 224 from non-inspected cheese. With regards to the isolation of *Pseudomonas* spp. confirmed by biochemical tests (oxidase positive and glucose negative), 47 isolates were identified, of which 24 were from inspected cheeses and 23 were from non-inspected cheese samples. The spoilage potential of these isolates is described in Table 1.

Of these total number of isolates, 85.9 and 91.5% of psychrotrophic bacteria and *Pseudomonas* isolated from the total number of samples of MFC, respectively, showed some type of spoilage potential. Moreover, there was a predominance of proteolytic and lipolytic potential simultaneously in nearly 60% of psychrotrophic and 70% of *Pseudomonas* spp. (Table 1)

Of the 47 isolates confirmed as *Pseudomonas* spp. by biochemical tests, 46 (97.9%) were confirmed by genus-specific PCR.

To verify the potential metalloprotease production capacity, a search of *aprX* was conducted individually in all isolates of this study, i.e. 694 psychrotrophic and 46 *Pseudomonas* spp. isolates proven by genus-specific PCR, totaling 740 reactions, as detailed in Table 2.

The 32 psychrotrophic isolates that displayed positive results in the assessment of *aprX* (Table 2) were also subjected to genus-specific PCR for *Pseudomonas* spp. Of these, 21 (65.63%) were confirmed, and the other 11 isolates were submitted to partial sequencing of the 16S rRNA for identification and phylogenetic analysis.

The identification of these 11 isolates that contained *aprX* were not identified as *Pseudomonas* is described in Table 3. Five genera were identified: *Serratia* spp. ($n=7$), *Raoultella ornithinolytica* ($n=1$), *Acinetobacter schindleri* ($n=1$), *Psychrobacter sanguinis* ($n=1$) and *Leuconostoc mesenteroides* ($n=1$). The species of the seven strains of *Serratia* were not identified because high percentages of similarity in the

Table 1. Spoilage potential of isolates of psychrotrophic bacteria, and the genus *Pseudomonas* isolated from 20 samples of inspected (10) and non-inspected (10) cheeses marketed in the municipality of Londrina, Paraná, from May to June 2017

Minas Frescal cheese	Group	Total number of isolates	Proteolytic and lipolytic	Proteolytic	Lipolytic	Non spoilage
		(n)	n (%)	n (%)	n (%)	n (%)
Inspected	Psychrotrophic	470	340 (72.4)	63 (13.4)	49 (10.4)	18 (3.8)
	<i>Pseudomonas</i> spp.	24	17 (70.8)	4 (16.7)	0 (0)	3 (12.5)
Non-inspected	Psychrotrophic	224	76 (33.9)	31 (13.9)	37 (16.5)	80 (35.7)
	<i>Pseudomonas</i> spp.	23	15 (65.3)	1 (4.3)	6 (26.1)	1 (4.3)
Total of samples	Psychrotrophic	694	416 (59.9)	94 (13.6)	86 (12.4)	98 (14.1)
	<i>Pseudomonas</i> spp.	47	32 (68.1)	5 (10.6)	6 (12.8)	4 (8.5)

Table 2. Production potential of metalloprotease (*aprX*) of psychrotrophic bacteria and of the *Pseudomonas* spp., and the spoilage potential of these isolates from inspected and non-inspected Minas Frescal cheese on agar plates

Minas Frescal cheese	Group	Total number of isolates	<i>aprX</i>	Proteolytic and lipolytic	Proteolytic	Lipolytic	Non spoilage
		(n)	n (%)	n (%)	n (%)	n (%)	n (%)
Inspected	Psychrotrophic	470	27 (5.7)	21 (77.78)	2 (7.41)	4 (14.81)	0 (0)
	<i>Pseudomonas</i> spp.	23	23 (100)	16 (69.57)	4 (17.39)	0 (0)	3 (13.04)
Non-inspected	Psychrotrophic	224	5 (2.2)	3 (60)	0 (0)	2 (40)	0 (0)
	<i>Pseudomonas</i> spp.	23	20 (86.96)	15 (75)	0 (0)	4 (20)	1 (5)
Total number of samples	Psychrotrophic	694	32 (4.61)	24 (75)	2 (6.25)	6 (18.75)	0 (0)
	<i>Pseudomonas</i> spp.	46	43 (93.5)	31 (72.10)	4 (9.30)	4 (9.30)	4 (9.30)

identity matrix (greater than 99%) were observed within several species of the 17 representative sequences of the genus available in RDP. The possible species indicated by a sequence representative of all isolates in the present study can be observed in Figure 1.

DISCUSSION

The highest mean score of psychrotrophic in the inspected cheeses compared to non-inspected cheeses can be explained by the greater control in storage, mandatory refrigeration (Brasil 1997), and strict observation in all inspected samples evaluated. Sangaletti et al. (2009) found that the psychrotrophic counts increase in MFC with the refrigeration storage period, increasing from 1.4×10^3 CFU/g on the first day after manufacturing to 4.5×10^{11} CFU/g after 30 days under these conditions. The lack of control or even the absence of refrigeration until the moment of commercialization of non-inspected cheeses can lead to higher counts of mesophilic microorganisms, but result in lower counts of psychrophilic bacteria.

As the formally marketed cheese samples already had, on average, 22 days of manufacture, and therefore, were kept under refrigeration, it is possible that the psychrotrophic counts would be lower if the samples were evaluated with a smaller storage period. In addition, the higher psychrotrophic counts in inspected cheese samples can be due to the inoculation of lactic acid bacteria (LAB) cultures in pasteurized milk intended for the production of cheese in dairy products. Several LAB cultures are also psychrotrophic and may demonstrate proteolytic and/or lipolytic activity (Ribeiro Júnior et al. 2018).

Pseudomonas is considered the main genus among psychrotrophic microorganisms in milk and dairy products (Ozturkoglu-Budak et al. 2016, Vithanage et al. 2016, Xin et al. 2017) and contamination can occur by the hands of milkers, and from the surface of the cows' udders and milking equipment, and from poorly sanitized refrigerated tanks (Vidal et al. 2017). From the average counts in the samples evaluated in the present study, however, it is possible to affirm that *Pseudomonas* spp. represent only 0.06% of the psychrotrophs (6.86×10^5 of 1.07×10^9 UFC/g) of the inspected Frescal Minas cheese samples and 0.46% (2.08×10^6 of 4.5×10^8 CFU/g) of the non-inspected ones evaluated in the present study. Other psychrotrophs than non-*Pseudomonas* can be more important for the Brazilian MFC quality and shelf-life, as reported by Ribeiro Júnior et al. (2018) in Brazilian raw milk.

Microorganisms of the *Pseudomonas* genus in raw milk are eliminated by pasteurization (Dogan & Boor 2003). Their isolation from inspected cheese samples can be due to recontamination after milk pasteurization, at any stage of production.

It is known that the low efficiency of cleaning and sanitizing of utensils (Gruetzmacher & Bradley 1999), the capacity of biofilm formation on equipment, temperature, storage time, (Hammad 2015) and the poor quality of water (Cousin & Bramley 1981, Fagundes et al. 2006) may influence the contamination of processed cheese samples by *Pseudomonas* spp. in industries. Therefore, additional cleaning and sanitizing measures for equipment should be implemented, which will uphold the quality of pasteurized milk, thus ensuring a better quality of the final product. The presence of *Pseudomonas* spp. in

Table 3. Genetic Identification and spoilage activity of psychrotrophic bacteria with expression potential of alkaline metalloprotease (*aprX*) not confirmed as *Pseudomonas* spp., isolated from inspected and non-inspected Minas Frescal cheese samples

Identification	No.	GenBank accession No.	Genetic similarity (%)	Cheese sample	Spoilage potential (No.)	Affiliation
<i>Serratia</i> spp.	7	MG932677	99.2-99.8	Inspected	Prot and Lipo (6) Prot (1)	Gammaproteobacteria
<i>Raoultella ornithinolytica</i>	1	MG932678	100	Inspected	Prot and Lipo	Gammaproteobacteria
<i>Acinetobacter schindleri</i>	1	MG932679	99.6	Inspected	Lipo	Gammaproteobacteria
<i>Psychrobacter sanguinis</i>	1	MG932680	99.8	Non-inspected	Lipo	Gammaproteobacteria
<i>Leuconostoc mesenteroides</i>	1	MG932681	100	Non-inspected	Lipo	Firmicutes

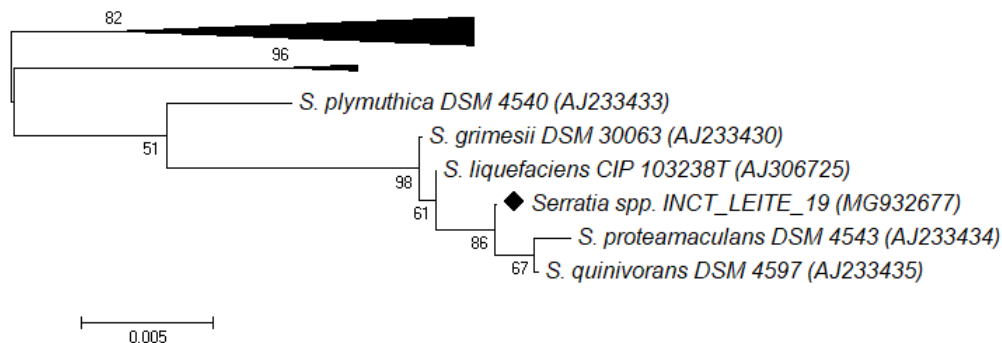


Fig.1. Phylogenetic tree of species of the genus *Serratia* (GenBank accession number) prepared using the alignment of 668bp of the 16S rRNA gene, Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates. The strain marked with a diamond symbol is representative of seven species of this genus isolated in this study. The bar indicates the percentage of nucleotide substitution.

non-inspected cheeses can be due to possible manufacturing with raw milk or environmental contamination at any point in the processing or marketing of the cheese.

A study by Sangaletti et al. (2009) demonstrated that 96.38% of psychrotrophic bacteria isolated from MFC showed lipolytic potential, and 78.17% showed proteolytic potential, which is as high as the value in the present study (Table 1).

Dogan & Boor (2003) isolated 338 strains of *Pseudomonas* spp. from raw milk and found that 51% were producers of proteases and 67% were producers of lipase, suggesting that the lipolytic and proteolytic activities varied among the different species of *Pseudomonas* spp. In these study the *Pseudomonas* are identify only at genus level. In the study by Hammad (2015), of 80 Domiati cheese samples collected, 70 were positive for *Pseudomonas* spp. Of the 80 isolates confirmed as *Pseudomonas*, 97.5% and 87.5% were potentially proteolytic and lipolytic, respectively. *P. fluorescens* was the most common species isolated.

The results of AprX production by *Pseudomonas* spp. in this study were higher than that in the study of Hammad (2015), who reported that 33 (41.25%) isolates of *Pseudomonas* spp. from cheeses were aprX positive.

It can be verified that not all the isolates that exhibit *aprX* express it constantly, since isolates that showed the *aprX* gene did not present proteolytic activity in plaques (Table 2). This intermittence in the expression of *aprX* may be conditioned by other factors, such as the availability of other substrates in the medium, as was also observed in another study (Ribeiro Júnior et al. 2018).

Serratia spp. are known psychrotrophs with a potential expression of AprX (Ribeiro Júnior et al. 2018). The present study identified all the isolates of this genus in samples of formally produced MFC (Table 3). This bacterium, as well as all other gram-negative bacteria, is not thermotolerant bacteria. Thus, it is possible to affirm that the contamination of cheeses by these micro-organisms occurred after pasteurization, i.e., during processing.

Despite displaying an expression potential of AprX, *Acinetobacter schindleri*, *Psychrobacter sanguinis*, and *Leuconostoc mesenteroides* showed no proteolytic activity on the plates, but only a lipolytic activity (Table 3). Thus, like strains of *Pseudomonas* spp., the expression of *aprX* may be related to other unknown factors.

The genus *Raoultella* was separated from the genus *Klebsiella* in 2001 (Drancourt et al. 2001). This is a known

human pathogen (Seng et al. 2016) and presents only four type sequences available in the RDP. In Figure 2, it is possible to observe that the isolate of the present study was grouped with the species *Raoultella ornithinolytica* and was also 100% compatible with this species in the identification by the identity matrix (Table 2). No previous reports of the production potential of AprX were found, but there is a description of a psychrotrophic proteolytic agent of milk in Slovakia (Pukančíková et al. 2016).

The genus *Acinetobacter* is a known component of spoilage-related microbiota of milk (Von Neubeck et al. 2015). These bacteria are psychrotrophs (Vithanage et al. 2016, Xin et al. 2017) and considered as emerging pathogens associated with human infections (Turton et al. 2010). The large percentage of identity matrix similarity (Table 3) observed in the strain isolated in this study with the species *A. schindleri* strain type LUH5232T (accession number AJ278311) can also be found in the phylogenetic proximity of the strains in Figure 3. No previous reports on the potential of AprX expression in *Acinetobacter* species or the isolation of *A. schindleri* species from cheeses were found.

P. sanguinis isolated in this study is reported to be an uncommon human pathogen (Le Guern et al. 2014) with a preference for cold (Rodrigues et al. 2009) and aquatic environments (Wirth et al. 2012). No previous reports of the isolation of this species in milk or cheeses, or their spoilage potential, were found. Delbès et al. (2007) found only the species *P. faecalis* in raw milk and cheeses in France. In Figure 4, one can observe the phylogenetic proximity of the strain isolated by the present work with the *P. sanguinis* type strain, with a similarity calculated at 99.8%.

Bacteria of the genus *Leuconostoc* are described as lactic acid bacteria (LAB) (Kleppen et al. 2012). Figure 5 indicates that the isolate from this study presents 100% of phylogenetic proximity with the type species *L. mesenteroides* (AB023247). This isolate was obtained from a sample of non-inspected cheese. Therefore, the possibility that this strain originates from some LAB culture used as a fermenting agent in cheese production is discarded, or rather, it is a component of the autochthonous milk microbiota. The expression potential of AprX by this strain is also relevant, since this enzyme is related to the Gammaproteobacteria class, according to Table 3.

A study by Ribeiro Júnior et al. (2018) also demonstrated that other psychrotrophic microorganisms from milk, besides *Pseudomonas* spp., may present a potential expression of AprX,

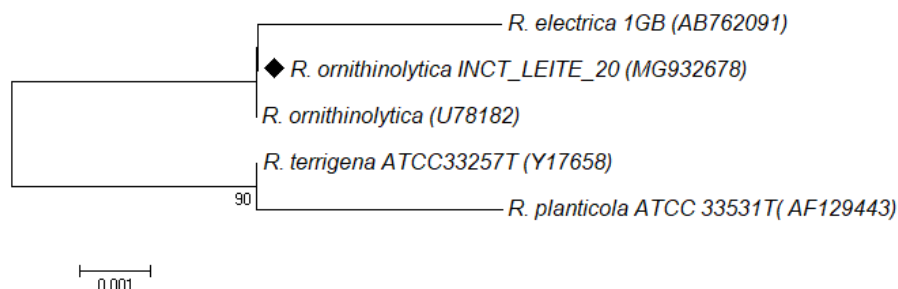


Fig.2. Phylogenetic tree of type sequences of species of the genus *Raoultella* (GenBank accession number) prepared using the alignment of 384bp of the 16S rRNA gene, Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates. The strain marked with a diamond symbol was isolated in this study. The bar indicates the percentage of nucleotide substitution.

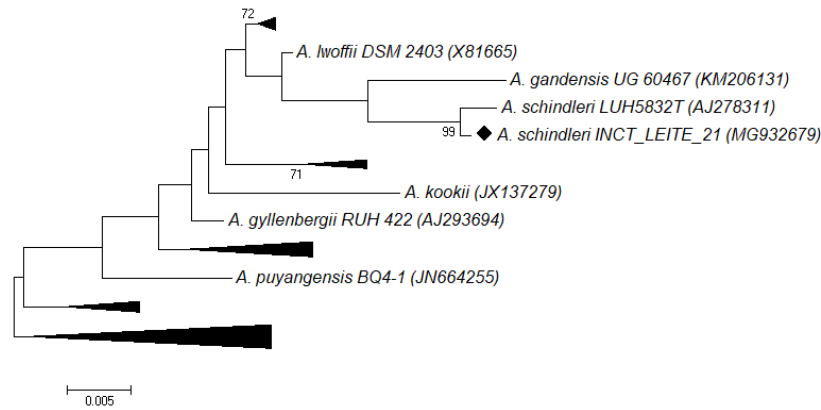


Fig.3. Phylogenetic tree of type sequences of species of the genus *Acinetobacter* (GenBank accession number) prepared using the alignment of 541bp of the 16S rRNA gene, Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates. The strain marked with a diamond symbol was isolated in this study. The bar indicates the percentage of nucleotide substitution.

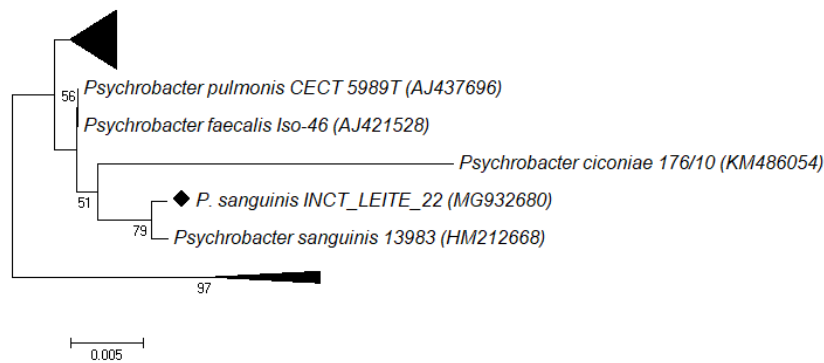


Fig.4. Phylogenetic tree of sequences of type species of the genus *Psychrobacter* accession (GenBank accession number) prepared using the alignment of 468bp of the 16S rRNA gene, Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates. The CEPA marked with diamond symbol was isolated in this study. The bar indicates the percentage of nucleotide substitution.

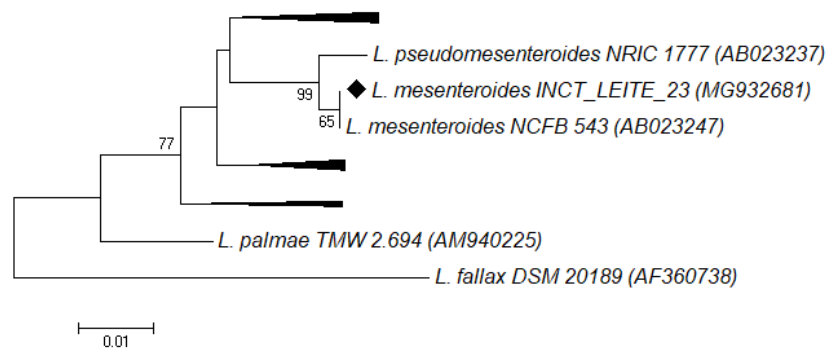


Fig.5. Phylogenetic tree of sequences of type species of the genus *Leuconostoc* (GenBank accession number) prepared using the alignment of 323bp of the 16S rRNA gene, Neighbor Joining method, Tamura-Nei model, and bootstrap support for 1000 replicates. The strain marked with a diamond symbol was isolated in this study. The bar indicates the percentage of nucleotide substitution.

such as *Serratia ureilytica*, *Enterobacter kobei* and *Yersinia enterocolitica*, all belonging to the Gammaproteobacteria class, in addition to *R. ornithinolytica*, *A. schindleri* and *P. sanguinis* identified in this study (Table 3). However, the isolation of *L. mesenteroides* (Bacilli) reveals that the dispersion of *aprX* can go beyond the Gammaproteobacteria class, broadening the spectrum of spoilage-related microorganisms of milk and dairy products.

CONCLUSIONS

Pseudomonas spp. and other potential spoilage-related psychrotrophs can be isolated in equivalent quantities, both in formally produced and commercialized and non-inspected MFC in Brazil.

The industrial production processes are not sufficient to control the contamination of MFC by these microorganisms, since the cheese possibly manufactured from raw milk present

lower counts of spoilage-related psychrotrophs than that industrially processed with pasteurized milk. Or, it is possible that the lack of refrigeration of the informal cheese was determinant of the low count of psychrotrophs in relation to the formal MFC.

Pseudomonas and other psychrotrophs isolated from MFC are, in their majority, simultaneously proteolytic and lipolytic, and may deteriorate the quality of the cheeses. Furthermore, microorganisms not yet described as cheese-spoilage bacteria may be emerging targets for quality control, and the factors that influence the expression and the dispersion of *aprX* gene between genera of psychrotrophs need to be elucidated.

The production of MFC with pasteurized milk should be safeguarded to avoid the risk to consumer health. In addition, industrial hygiene practices should be followed to ensure cheese production with less contamination by spoilage microorganisms.

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Conflict of interest statement. The authors declare that they have no conflicts of interest.

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***Salmonella* spp. prevalence, antimicrobial resistance and risk factor determination in Colombian swine farms¹**

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ABSTRACT.- Giraldo-Cardona J.P., Guadrón-Ramírez D., Chamorro-Tobar I., Pulido-Villamarín A., Santamaría-Durán N., Castañeda-Salazar R., Zambrano-Moreno C. & Carrascal-Camacho A.K. 2019. *Salmonella* spp. prevalence, antimicrobial resistance and risk factor determination in Colombian swine farms. *Pesquisa Veterinária Brasileira* 39(10):816-822. Departamento de Microbiología, Facultad de Ciencias, Pontificia Universidad Javeriana, Carrera 7 # 43-82, Ed. 52, Bogotá, Colombia. E-mail: adriana.pulido@javeriana.edu.co, acarrasc@javeriana.edu.co

To determine *Salmonella* spp. prevalence/seroprevalence, antimicrobial resistance patterns and risk factor identification associated with its presence in Colombian swine farms. 504 samples (Faeces, swabs and environment samples) were obtained from 21 farms distributed in four geographical regions in Colombia. *Salmonella* spp. microbiological and molecular detection were determined by two *Salmonella* spp. MDS3M™ and MALDI-TOF MS assays, respectively. In addition, for serological evaluation 231 serum samples were analyzed employing ELISA *Salmonella* Pigtype®-*Salmonella* Ab (QUIAGEN®). Additionally, 41 isolates were tested for antimicrobial susceptibility using broth microdilution technique (Panel B1016-180 Beckman Coulter NC72®) and verified with WHONET 2016 software. Risk factors were assessed from a survey and analyzed for statistical significance by U Mann-Whitney test. An 8.9% prevalence (n=45) and 38.1% (n=88) seroprevalence were determined. All isolates presented 100% antimicrobial susceptibility against amikacin. However, resistance against penicillin, tetracycline, cefuroxime and trimethoprim/sulfamethoxazole was present in more than 50% of evaluated strains. Risk factors associated with *Salmonella* spp. presence were surface water use, rough-surfaced on floors, presence of hoppers as feeders and worker's boots. Bacteria were present in animals and environmental samples from evaluated farms. Animal contact and/or exposure with the microorganism were also evident in obtained serological response. Bacteria presence depended on management practices and infrastructure, likewise antibiotic use, supplemented in the diet may have induced an increase in *Salmonella* spp. antimicrobial resistance.

INDEX TERMS: *Salmonella* spp., antimicrobial resistance, risk factors, swine farm, prevalence, seroprevalence, susceptibility test, pigs, Colombia.

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RESUMO.- [*Salmonella* spp. prevalência, resistência antimicrobiana e determinação de fatores de risco em granjas suínas colombianas.] Para determinar *Salmonella* spp. prevalência/soroprevalência, padrões de resistência antimicrobiana e identificação de fatores de risco associados à sua presença em granjas suínas colombianas. Foram obtidas 504 amostras (fezes, zaragatoas e amostras do ambiente) de 21 fazendas distribuídas em quatro regiões geográficas da Colômbia. *Salmonella* spp., a detecção microbiológica e molecular foi determinada por 2 *Salmonella* spp. Ensaios

MDS3M™ e MALDI-TOF MS, respectivamente. Além disso, para avaliação sorológica, foram analisadas 231 amostras de soro empregando ELISA Salmonella Pigtype® - Salmonella Ab (QUIAGEN®). Além disso, 41 isolados foram testados quanto à suscetibilidade antimicrobiana usando a técnica de microdiluição em caldo (Painel B1016-180 Beckman Coulter NC72®) e verificados com o software WHONET 2016. Os fatores de risco foram avaliados em uma pesquisa e analisados quanto à significância estatística pelo teste U Mann-Whitney. Foram determinadas prevalências de 8,9% (n=45) e 38,1% (n=88). Todos os isolados apresentaram 100% de suscetibilidade antimicrobiana à ampicilina. No entanto, resistência à penicilina, tetraciclina, cefuroxima e trimetoprim/sulfametoxazol estava presente em mais de 50% das cepas avaliadas. Fatores de risco associados à *Salmonella* spp., presença de uso de água de superfície, superfície áspera no chão, presença de tremonhas como alimentadores e botas de trabalho. Bactérias estavam presentes em animais e amostras ambientais de fazendas avaliadas. O contato animal e/ou a exposição ao microrganismo também foram evidentes na resposta sorológica obtida. A presença de bactérias dependia de práticas de manejo e infraestrutura, assim como o uso de antibióticos suplementados na dieta pode ter induzido um aumento de *Salmonella* spp. resistência antimicrobiana.

TERMOS DE INDEXAÇÃO: *Salmonella* spp., suinocultura, prevalência, soroprevalência, teste de suscetibilidade, fatores de risco, suínos, Colômbia.

INTRODUCTION

Swine production is a growing industry in Colombia. It is reflected by the 1,064,555 heads of pigs sacrificed during the third trimester of 2017 (DANE 2018). Additionally, consumption of pork meat “*per capita*” is estimated in 9.3kg/year (Porkcolombia 2018), moreover porcine population in 2017 was established as 5,327,460 animals, mainly located in the departments of Antioquia (34.53%), Cundinamarca (9.24%), Córdoba (6.9%), Valle del Cauca (5.82%) and Meta (4.19%) (ICA 2017).

Swine production is likely affected by different pathogens, such as viruses, parasites and bacterias. Pathogens generate serious health problems and economic losses resulting from growth rate variation, decrease in weight gain, meat quality alteration, and occasional animal death. In addition, veterinary medical care costs, required diagnosis tests and treatment costs (Hurd et al. 2002).

Pathogen presence may represent a public health risk, since some cause zoonoses. Additionally, certain pathogens can increase human mortality rate, where *Salmonella* spp. is the main microorganism related with this problem (Baer et al.

2013). The United States presents approximately 80.3 million cases of salmonellosis per year, resulting in 150.000 deaths approximately (Majowicz et al. 2010). On the other hand, the European Food Safety Authority (EFSA) reported that in Europe 8.9% of salmonellosis cases were attributed to pork consumption (EFSA 2016). In Colombia, from 2003 the “Instituto Nacional de Salud” (INS) obtained 7,424 *Salmonella* spp., isolates from human clinical samples (97.2%) and water and food samples (2.8%), none the less the exact source of origin was not specified (INS 2011).

Salmonella spp. is a pathogen that has the ability to persist in the environment for long periods of time, causing asymptomatic infections. Therefore, infected animals can be a source of contamination for the healthy population of the herd and even in slaughterhouses (Martín-Peláez et al. 2010, Baer et al. 2013). Then, one strategy is to interrupt dissemination, using prevention and control measures, since the first source of infection is the farm (EFSA 2006). Furthermore, frequently microorganisms express resistance to antibiotics, specifically *Salmonella* spp. strains isolated from the animal productive chain (Gomes-Neves et al. 2014).

In Colombia, *Salmonella* spp. presence has been shown in porcine meat juice; in slaughterhouse in Bogotá, with a 27.2% prevalence, whereas in the department of Tolima reported pork meat prevalence has been of 4.3% (Mora 2003, Ávila et al. 2013). Previous findings by GBAI research group from slaughterhouse sampling carried-out in 14 regions of the country have established prevalences of 12% and 28.2% in meat and mesenteric nodes, respectively (Ayala-Romero et al. 2018). However, presence and prevalence of *Salmonella* spp. in Colombian farms is unknown. Therefore, the aim of the present study was to determinate the prevalence/seroprevalence of *Salmonella* spp., antimicrobial susceptibility patterns and identify risk factors associated with its presence in swine farms in four regions of Colombia.

MATERIALS AND METHODS

Animal care and welfare. This study was performed under field conditions in commercial farms. Its housing and care were adequate. This project was approved by the institutional animal care Document No.C-077-16 (FUA 038-16) in “Pontificia Universidad Javeriana”.

Pig farms and population of study. According to the 2016 porcine census farms were sampled for breeding (19%) and full production cycle (replacement-females, pregnant sows, lactating sows, weaning, nursery, farrowing, grow finish or fattening pigs, and boars) located in areas of highest porcine production in the country Antioquia, Valle del Cauca, Eje Cafetero and Cundinamarca-Meta (Table 1).

Questionnaire survey. This study applied a survey to assess each farm on general farm conditions, pens, biosecurity measures,

Table 1. Location and type of farm analyzed

Department	Feeder pig production farm/ breeding farm	Full cycle farm	Number of farms
Antioquia		9	9
Valle del Cauca	1	2	3
Eje cafetero (Risaralda-Quindío-Caldas)	2	1	3
Cundinamarca-Meta	1	5	6
TOTAL	4	17	21

sanitary and environmental aspects, animal care, pig feeding and worker's occupational health among other aspects.

Population of study and sample size. Sampling was carried-out in a stratified manner and by proportional sampling for participating departments, using program sample size 1.5, with an estimated prevalence of 50%. Sample size was 395, however, 504 samples were obtained, after determining ideal sample size. Additionally, four farms volunteered to participate in the project. Therefore, a total of 21 farms enrolled in the study. The Table 2 details the type of samples obtained. Samples were obtained by veterinarian, following biosecurity measures and guidelines established by the world organization for animal health manual regarding land animals (chapter 1.1.1, section A, numeral 1). Also, packing and transport recommendation was followed as described in section D chapter 1.1.1 of the same manual (OIE 2004).

Fecal samples in pens. Five pens housing growing finish pigs were selected at random and feces were collected to pool approximately 25g of feces (Rajic et al. 2005).

Rectal swabbing. Obtained from pigs at different stages of production through the use of culturettes (Transytem®) with Clary-Blair transport medium (Wilkins et al. 2010).

Environmental swabbing. For each farm samples were obtained from a sponge, previously dampened in 15mL buffered peptone water (BPW) and rubbed over surfaces to obtain the sample (workers boots, pig pen drains and empty pens after cleaning and disinfection processes). In addition, approximately 500mL of water supply system was collected (Wilkins et al. 2010).

Total blood. Jugular vein blood samples were collected from animals at different phases of their productive cycle, using vacutainer system in tubes without anticoagulant (PuthVacumine®) (van der Wolf et al. 1999).

Sample processing. For *Salmonella* spp. isolation and identification, samples were microbiologically enriched in BPW. A 1/10 BPW dilution was performed for subsequent molecular detection.

Fecal samples in pens. 10g sample was weighed and 90mL BPW was added. Sample was incubated at 35°C for 18 to 24 hours (Wilkins et al. 2010).

Rectal swabbing. Initial non-selective processed in 5mL of BPW and incubated at 35°C for 18 to 24 hours.

Environmental swabbing. BPW dampened sponges rubbed against surfaces (workers boots, empty pens and pig pen drains) were initially nonselectively pre-enriched in 60mL BPW and incubated at 35°C for 18 to 24 hours. Collected water samples were processed according to the American Public Health Association (APHA) protocol section 9260b. As previously indicated, microbiological enrichment processed molecular detection using Molecular detection assay 2 *Salmonella* spp. (MDS 3M™).

Serology. Collected blood samples were centrifuged at 3,000rpm for 5min to obtain serum. Serum were processed using indirect ELISA employing the *Salmonella* kit Pigtype®-Salmonella Ab (QUIAGEN®) following manufacturer's instructions.

Identification. Positive samples obtained from the molecular detection system were recovered in agar Chromagar® *Salmonella*. Identification was confirmed by MALDI-TOF MS methodology (Bruker Daltonics).

Antimicrobial susceptibility testing. Taking into account the microorganism's zoonotic potential, antimicrobial susceptibility was evaluated against antibiotics used in human therapy. To this end, broth microdilution technique was used using Panel B1016-180 (Beckman Coulter; Negative Combo 72, NC72), as recommended by the Clinical and Laboratory Standards Institute (CLSI) M100-S27 (CLSI 2017). For data analysis Who-Net 2016 program was used.

Data analysis. Data was analyzed using descriptive statistics. Serological tests were performed following manufacturer's indications. A sample was considered positive when CP OD ≥ 0.7 and M/P Relation ≥ 0.3 . To establish the relationship between prevalence data and identified determined factors in the questionnaire, a Mann-Whitney U Test was carried-out using SPSS® software (IBM Company). Additionally, susceptibility tests were interpreted based on cut-off points by Who-Net program (Table 3).

RESULTS

Of the 504 analyzed samples 8.9% (n=45) were positive for *Salmonella* spp. as assessed by microbiological isolation and molecular identification. From the 231 serums evaluated a general seroprevalence of 38.1% (n=88) was evident. Prevalence

Table 2. Type of sample, sample quantity and type of analysis

Type of sample	Sample quantity	Type of analysis
Fecal samples in pens	5	Detection
Rectal swabbing	11	Microbiological/molecular (n=504) and
Empty pen swabbing	2	Susceptibility test (n=41)
Worker boots swabbing	2	
Pig pen drain swabbing	2	
Water	1 1(500ml collecting area) 1 11 (500ml feed water system)	
Total blood	11	Serology (ELISA) (n=231)

Table 3. Cut off points detected in antimicrobial susceptibility tests to cefotaxime (CTX), ampicillin (AMP), ciprofloxacin (CIP), colistin (COL) and trimethoprim/sulfamethoxazole (SXT)

Antibiotic	Cut off points	%R	%I	%S	%R 95%I.C.
CTX	S<=1 R>=4	5.6	25	69.4	1.0 - 20.1
COL	S<=2 R>=8	27.8	8.3	63.9	14.8 - 45.5
AMP	S<=8 R>=32	33.3	0	66.7	19.1 - 51.0
CIP	S<=.064 R>=1	11.1	5.6	83.3	3.6 - 27.0
SXT	S<=2 R>=4	50	0	50	33.2 - 66.8

and seroprevalence for each region analyzed was determined (Fig.1), as well as for each productive stage (Fig.2).

According to sample type, total prevalence in feces was 7.6% (8/105). The lowest prevalence observed was from rectal swabbing with 8.7% (8/231), followed by its presence in workers boots (9.5%) and pig pen drains 11.9% (5/42). The highest observed prevalence came from water samples 14.3% (3/21).

Positive samples, 45 in total were confirmed by molecular system detection, however the microbiological recovery was possible in 41 samples (91%). From these, antimicrobial susceptibility tests showed that 100% (41) of the strains were susceptible to amikacin, while 94.4% (39) were resistant to penicillin (P4), 94.4% to tetracycline (39), followed by 87.8% to cefuroxime (36), 52.8% to cephalothin (21), 48.7% (20) to trimethoprim/sulfamethoxazole and 11.2% to ciprofloxacin (5). Of the positive strains 12% displayed multi-resistance, i.e. resistance to at least minimum four antibiotics.

Risk factors in the farm associated with presence of *Salmonella* spp. were: the source of water (surface water type) ($P<.005$), type of floor as a porous surface in pig pens ($P<.030$), hopper type of feeder ($P<.005$) and workers boots ($P<.005$).

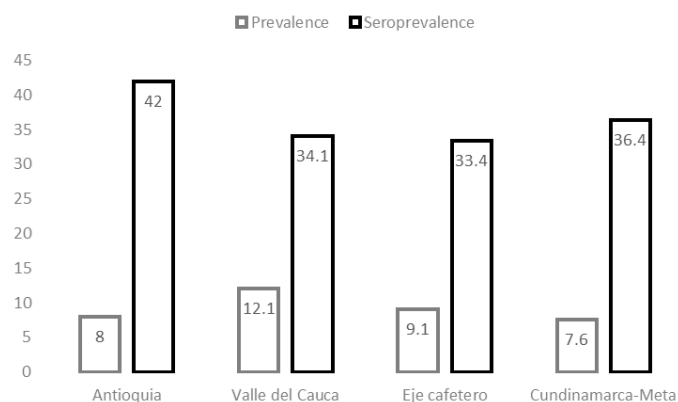


Fig.1. Percentage of prevalence and seroprevalence for *Salmonella* spp. by analyzed region.

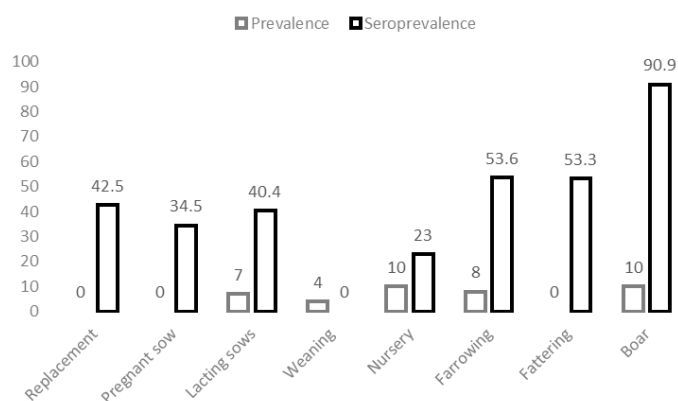


Fig.2. *Salmonella* spp. prevalence and seroprevalence percentage by analyzed stages of production

DISCUSSION

In 2010 in Canadá 36% single prevalence for pig *Salmonella* spp. was estimated (Wilkins et al. 2010), 12% in the Netherlands (van Der Wolf et al. 1999), while in Spain reported prevalence was 43% (García-Feliz et al. 2007). In contrast, lower prevalences have been described in Denmark 2.1% and Norway between 1 and 4% (Stege et al. 2000, Sandberg et al. 2002). Lower prevalences in this study could be accounted for by good porcine practices (GPP) in Colombian swine farms. However, obtained results did evidence bacteria presence in animals and in environmental samples from analyzed farms. Additionally, animals were in contact and/or exposed to the microorganism, as evidenced from serological response.

According to Funk et al. (2001) the productive stage influences pathogen presence variation. It has been reported that *Salmonella* spp. prevalence increases along with the growth stages, until reaching the fattening (grow-finish) stage (Funk et al. 2001). Several reports revealed a higher prevalence (57%) during fattening; however, results obtained in this study did not detect bacteria presence in this stage (Korsak et al. 2003, Dorr et al. 2009). Prevalence in pregnant sows and piglets was 7% and 4%, respectively. Different authors agree that pregnant sows are often more vulnerable to infection compared with piglets (Wilkins et al. 2010). Wilkins et al. (2010) reported prevalence differences between pregnant sows and piglets, where 59% pregnant females were positive, while only 32% were positive; data differing from the observed in this work for the two age groups. Nevertheless, it is important to take into account sample size difference, and the type of farms analyzed. It should be noted that although in some stages of the productive cycle isolates were not achieved, presence of serologically detected antibodies, suggest females could be in contact with the microorganism at some point of the cycle. Nevertheless, it might be indicative of effective control strategies implemented in evaluated farms at the particular stage of the cycle.

Differences in prevalence values of each stage of production might be justified by multiple variables, such as the geographical region where sampling took place, automation degree, production system and management practices in each farm (Baer et al. 2013). The most common implemented breeding management practices for swine production farms are All-In/All-Out (AIAO) system and Three-site swine production system (Dors et al. 2015). The first practice is used in Colombia, specifically in the farms analyzed. Thus, in agreement with Dors et al., implementation of this pig farm management method is a factor reducing *Salmonella* spp., presence (Dors et al. 2015).

With regards to general seroprevalence determined in the present work (38.1%) it was different from that reported in Mexico, Italy, USA and Spain, where lower seroprevalences were determined (28.7%, 19.3%, 5% and 4%, respectively) (Vicente et al. 2002, Montagnaro et al. 2010, Thakur et al. 2011, Pérez-Rivera et al. 2017). Nevertheless, for Cundinamarca-Meta, seroprevalence was 36.4%, a value close to 40% the seroprevalence reported by Pulido-Villamarín et al. (2016). In relation to results obtained for each stage group, through microscopic agglutination test (MAT) in South Korea a seropositivity of 46.7% was detected in females, 6.7% on farrowing and 3% in nursery (Vicente et al. 2002), similar values were observed in different female groups in this work. Nevertheless, this was not the case for nursery animals and

farrowing, where very dissimilar values were observed compared with those reported by the Korean report. This may be related with differences in biosecurity measures and management for each country, given sociocultural, economic and even environmental conditions specific to each one, as well as the methodologies used in each work.

In relation to the clinical samples, 7.6% prevalence was observed for fecal samples and 8.7% from rectal swabbing samples. Data was in agreement with reports in Canada, where a similar work detected the pathogen on the same type of sample, yet with an average prevalence of 25.2% (Rajic et al. 2005, Wilkins et al. 2010). Canadian reported prevalence was significantly higher compared with the value observed in the present work. Even though it has been established that bacteria isolates from animal clinical samples can be difficult to obtain given low concentrations, competition and overgrowth by other bacteria of the *Enterobacteriaceae* family, collectively it was possible to isolate the microorganism from this type of sample.

Presence of bacteria in the environment may be associated with the capacity to survive and persist for long periods of time (Baer et al. 2013). Some environmental samples, such as empty pens, drains and workers boots can harbor microorganisms, and might be an important source of indirect contamination within the farm (Wilkins et al. 2010). Furthermore, surface water presented the greatest number of isolates, suggesting a possible pathogen dissemination source within farms, since surface water is used for different activities, such as cleaning facilities, farm equipment, and drinking water, among others. This result may be associated with *Salmonella* spp. capacity to establish biofilms and colonize pipes for water distribution to different sites at the farm (Yang et al. 2015).

González et al. (2015) claimed presence of this microorganism in water drinkers is associated with deficiencies in cleaning and disinfection procedures, hence promoting dissemination to the non-infected population. It is also important to consider at the moment of washing the pens, some fecal material contained within the pen can splash and contaminate drinkers. Therefore, other aspects to consider are the design of drinkers. It has been described that basin type drinkers reduce the risk of *Salmonella* spp. infection compared with fountain type or wells (Bahnsen et al. 2006). However, in this work all the farms evaluated (100%) employ nipple drinkers. Hence, it is possible microorganism presence resulted from contaminated water, together with ineffective maintenance and deficiencies in disinfection protocols, contributing with pathogen dissemination and persistence. *Salmonella* spp. dissemination through water increases when water is obtained from wells and surface water without purification, such waters can be contaminated by healthy carriers wildlife passing, from nearby farms and even from the same farm, as could be the case for farms evaluated in this study (Mejia et al. 2006), where 76% of the farms are located near other livestock farms, mostly cattle, species known to carry bacteria and thus contaminate water bodies.

Another variable resulting in *Salmonella* spp. presence and dissemination in the farm are pen drainage systems, the 11.9% bacteria presence was detected, where contamination was attributed to sewage water, which can contain infected pig feces remaining in the drainage system. Additionally, these are contamination sites, since they attract insects and rodents,

which can be bacteria carriers and disseminators, allowing their continuous presence and cross-contamination in the pen. On the other hand, 7.1% bacteria were also detected in previously disinfected empty pens, which could indicate deficiencies in the procedure. Among the many causes could be the chemical properties of the disinfectant used, its concentration, contact time, pen surface or type of floor. In any case, failures in cleaning processes could have favored inactivation of the product, even the use of high pressure hoses can generate aerosol, disseminating the pathogen within the facilities (Dors et al. 2015, Montagnaro et al. 2010). In turn, farms where empty pens were only cleaned with water, a direct correlation was observed between the type of floor and *Salmonella* spp. presence ($P < 0.05$), specifically bacteria can adhere to the floor surface or lodge in floor pores, without achieving an effective pathogen elimination, given the lack of appropriate disinfectants that favor microorganism elimination.

In this work 66.7% of the farms had mixed floors and 28.7% cement floors. However, the type of floor changes according to production stage. This findings, are in agreement with Andres & Davies (2015) report in who claim that floor design influences the way waste can be removed, and microorganism persistence after cleaning and disinfection processes (Andres & Davies 2015). Even though these risks are a fact, procedures, such as flaming and rotation of disinfectants could prevent bacteria from attaching. Although some farms in this work carry out such procedures, untreated water is still used, thus favoring pathogen presence.

Furthermore, worker's boots can be cross-contamination agents, as was detected in this work, since bacteria presence in these elements was 9.5%. All evaluated farms in this study had footbaths for boot disinfection. However, previous unsuitable cleaning results in feces collection in sole furrows, and contact with this organic matter with products, inactivates the disinfectant (Amass et al. 2000, Pritchard et al. 2005, Wales et al. 2011, Rabie et al. 2015).

In agreement with microbiological findings, one management variable associated with seropositivity was the frequency with which manure was collected. This task is performed on a daily basis, being the most frequent activity. According with reports, it is essential to remove feces from the environment, since *Salmonella* spp. is spread by the fecal-oral route. Therefore, farm hygiene practices are directly related with bacteria permanence in the environment, as well as increase in infected animals (Xiao et al. 2005, Nielsen 2013). Although manure collection contributes with bacterial load reduction, use of untreated water, allows pathogen recirculation. Another statistically significant variable observed in this study was the fecal pit, which is a source of attraction for flies that become vectors of the microorganism (Béjar et al. 2006).

In relation to antimicrobial resistance from the isolates obtained, it was established that 57% of analyzed farms added antibiotics and other supplements to food. As was reported by Diaz et al. (2011) our findings are consistent with their report stating in Colombia is common to formulate concentrate with antibiotics in swine farms. Moreover, according to the "Instituto Colombiano Agropecuario" (ICA) reports pigs are treated with ciprofloxacin, ampicillin and trimethoprim/sulfamethoxazole for different purposes including preventive. These same products are used therapeutically in human salmonellosis.

Hence, resistance to the antimicrobial by the microorganism can arise, due to prolonged and often exposure to the antibiotic.

According to Butaye et al. (2003) the main cause of resistance in isolate strains from farms is attributed to indiscriminate and excessive use of antibiotics in animal rations, using them as growth promoters, thus inducing multiresistance. Additionally, plasmid transfer among bacteria found in swine gastrointestinal tract is facilitated. Multiresistance data obtained in this work is in agreement with Gomes-Neves et al. (2014) report who observed 63% of analyzed isolates were multiresistant. Furthermore, as aforementioned it is necessary to emphasize the resistance to first and second choice antibiotics used in human therapy against salmonellosis (trimethoprim/sulfamethoxazole, ampicillin, colistin, cefatoxime, and ciprofloxacin), of which in the present investigation a significant percentage of resistance was obtained against trimethoprim/sulfamethoxazole and to ciprofloxacin, this added to the fact that 5.6% of the isolates showed shared resistance to both antibiotics.

Finally, data obtained for antimicrobial resistance in farms included in this work, revealed the presence of *Salmonella* spp. strains resistant to first line antibiotics and multidrug-resistance strains, which can have a negative impact on national public health. Therefore, it is necessary to establish an integrated monitoring program for epidemiological surveillance, with the objective to decrease zoonotic risk.

CONCLUSIONS

Although in Colombia good porcine practices are established for porcine production, it is evident bacteria were present in animals and environmental samples of analyzed farms.

Animal contact/exposure to the microorganism was also evidenced by serological response obtained.

The presence of these bacteria was influenced by management practice (type of water used, feeders-hopper type, handling by workers) and infrastructure (porous floor) in analyzed farms.

Use of antibiotic supplements in food could be inducing high antimicrobial resistance against *Salmonella* spp.

Emergence of multiresistance strains is becoming more frequent and with a tendency to increase.

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Medical therapy using omeprazole in 12 hydrocephalic dogs: clinical, diagnostic, and therapeutic findings¹

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ABSTRACT. Pelegrini L.F., Silva N.F., Campos O.P.S., Nery C.C., Silva F.M., Lemos R.S., Yamauchi K.C.I. & Amude A.M. 2019. **Medical therapy using omeprazole in 12 hydrocephalic dogs: clinical, diagnostic, and therapeutic findings.** *Pesquisa Veterinária Brasileira* 39(10):823-829. Setor de Clínica Médica de Pequenos Animais, Serviço de Neurologia Animal, Neurologia Clínica e Cirúrgica, Faculdade de Medicina Veterinária, Universidade de Cuiabá, Av. Beira Rio 3300, Cuiabá, MT 78065-420, Brazil. E-mail: amamude@hotmail.com

According to experimental studies with healthy dogs, omeprazole might decrease the CSF production by about 26%; therefore, book texts have been suggested the usage of omeprazole in medical protocols for hydrocephalus treatment. However, to the best knowledge of the authors, the usage and medical response of the omeprazole with substantial group of illness dogs, such as hydrocephalic animals, was lacking. This report describes clinical, diagnostic, and therapeutic findings in 12 dogs with hydrocephalus in which omeprazole were used for medical treatment. The diagnosis of hydrocephalus was accomplished by transcranial sonography (TCS) and/or computed tomography. The ventricular measurement was assessed periodically by TCS during medical treatment. Six dogs were diagnosed with non-obstructive hydrocephalus and in the other 6 cases hydrocephalus occurred with other concomitant anomalous encephalic disease often related with obstructive hydrocephalus, such as quadrigeminal cist, arachnoid cyst, chiary-like malformation, and syringomyelia. All of them had medical improvement after the use of omeprazole and the most of the cases had ventricular size reduction. In 10 dogs, the omeprazole was used as single drug, and in 2 dogs medical treatment with steroids and/or diuretics was previously being performed, and omeprazole was added because conventional treatment was resulting in mild to unsatisfactory medical control of the neurological status. The results of this paper shown that omeprazole may be used to ameliorate the neurological status in symptomatic hydrocephalic dogs. This work may represent the first description about the use of omeprazole in order to treat a substantial group of affected dogs with suspected increased intracranial pressure by hydrocephalus, probably due to limitation of CSF production.

INDEX TERMS: Medical therapy, omeprazole, hydrocephalus, dogs, clinic, diagnostic, therapeutic findings.

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RESUMO. [Terapia médica utilizando omeprazol em 12 cães hidrocefálicos: achados clínicos, diagnósticos e terapêuticos.] O omeprazol diminui a produção do fluido cerebrospinal (FCE) por cerca de 26% de acordo com estudos experimentais em cães saudáveis. Segundo o conhecimento dos autores, embora utilizado na prática clínica e recomendado em livros textos, não há até o momento estudos clínicos em um grupo substancial de animais avaliando a resposta terapêutica ao uso do omeprazol em pacientes enfermos, tais como cães hidrocefálicos sintomáticos. Este trabalho descreve os achados clínicos, diagnósticos e terapêuticos em 12 cães com hidrocefalia que foram submetidos ao tratamento com omeprazol para o manejo médico de hidrocefalia. O diagnóstico de hidrocefalia e doenças neurológicas concomitantes foi realizado por

ultrassonografia transcraniana (USTC) e/ou tomografia computadorizada. A mensuração do tamanho ventricular foi realizada pela USTC durante o tratamento médico. Seis cães foram diagnosticados com hidrocefalia não obstrutiva e os outros 6 casos apresentaram hidrocefalia concomitante com outras afecções encefálicas anômalas comumente associada à hidrocefalia obstrutiva, tal como cisto quadrigêmio, cisto aracnóide, síndrome de *chiari-like* e seringomegalia. Em 10 cães o omeprazol foi utilizado como droga única e em 2 cães a terapia inicial foi a convencional utilizando esteroides e diuréticos, e o omeprazol foi adicionado, pois a resposta clínica a terapia convencional foi insatisfatória. Todos os animais obtiveram melhora dos parâmetros neurológicos e a maioria teve uma redução do tamanho ventricular após o uso do omeprazol. Os resultados deste estudo demonstram que o omeprazol pode ser utilizado para melhorar o estado neurológico em cães com hidrocefalia. Este estudo representa a primeira descrição clínica usando o omeprazol para tratar uma série de cães com suspeita de aumento da pressão intracraniana devido à hidrocefalia, provavelmente pela capacidade do fármaco em limitar a produção do FCE.

TERMOS DE INDEXAÇÃO: Terapia, omeprazol, hidrocefalia, cão, clínica, diagnóstico.

INTRODUCTION

Hydrocephalus is a condition involving an increase in the volume of the cerebrospinal fluid (CSF) with abnormal dilatation of the ventricular system within the cranium and consecutive destruction and atrophy of surrounding nervous tissue of the brain. The choice of treatment is generally dictated by the physical status, the age of the animal, and the cause of the hydrocephalus (Thomas 2010, Estey 2016). Ventricle-peritoneal shunts have been commonly used to divert the flow of CSF from the ventricles of the brain to the peritoneal cavity in human patients with hydrocephalus, and have proved to be an effective surgical treatment for hydrocephalus in dogs and cats (Kim et al. 2006, Kitagawa et al. 2008, Schmidt et al. 2019). Surgery does not decrease the damage to the nervous tissue secondary to the increased intracranial pressure (ICP) that have already occurred at the moment of diagnosis; in addition, surgery might be indicated with criteria because of the risk of the anesthesia, the risk of neurological and systemic complications intraoperative, and the risk of postoperative complications of shunt placement (Platt & Olby 2004, Dewey 2008, Gradner et al. 2019). No significant differences have been noted in clinical or imaging findings between surgically and medically treated dogs (Gillespie et al. 2019). In addition, ventricle-peritoneal shunts have an elevated cost, limiting this surgery for hydrocephalus treatment by many owners. Therefore, medical treatment has been commonly used to manage ICP in dogs with hydrocephalus (Platt & Olby 2004, Dewey 2008, Thomas 2010, Gillespie et al. 2019).

Medical treatment of hydrocephalus includes general supportive care and the administration of medications to restrict the production of cerebrospinal fluid and hence reduce the ICP. Angiotensin II plays an important role in regulation of blood flow to the choroid plexus and decreases the rate of production of CSF in rabbits and rats (Maktabi et al. 1990, 1993, Chodowski et al. 1995). Ethanol at a concentration of 150mg/dl (far below lethal levels) is one of the most potent

inhibitory drugs for decreasing CSF production in dogs (Javaheri & Corbett 1998). However, therapeutic uses of these drugs to treat hydrocephalus in dogs are not available.

Different medications has been traditionally used with this propose such as steroids (glucocorticoids), furosemide, mannitol, and carbonic anhydrase blockers (acetazolamide) (Sahar & Tsipstein 1978, Pollay et al. 1983, Platt & Olby 2004, Dewey 2008, Kim et al. 2009, Park et al. 2009, Estey 2016). Furosemide and acetazolamide may decrease the CSF production. The primary mechanism of action for various types of glucocorticoids is to decrease cerebrospinal fluid production; prednisone is the drug of choice and dexamethasone sodium phosphate has also been used to treat hydrocephalus, but long-term use should be avoided because of the higher incidence of side effects (Platt & Olby 2004, Dewey 2008, Thomas 2010, Estey 2016). Mannitol has been used mainly in cases of rapidly decompensating hydrocephalus (Thomas 2010), due its osmotic gradient (Pollay et al. 1983) and may be not used as on a daily basis therapy (Estey 2016).

Many of the drugs used to treat hydrocephalus (furosemide, mannitol, and carbonic anhydrase blockers) have diuretic activity, and caution must be exerted when using any drug with diuretic activity because electrolyte depletion is a common sequel; in addition, electrolyte loss is accelerated when diuretics are used in combination with steroids (glucocorticoids). Acetazolamide combined with glucocorticoids may cause potassium depletion at the therapeutic drug concentration, which often requires discontinuation of acetazolamide (Platt & Olby 2004, Dewey 2008, Thomas 2010, Estey 2016).

Experimentally, omeprazole was already proved to reduce ICP by decreasing cerebrospinal fluid production in healthy rabbits (Lindvall-Axelsson et al. 1992) and dogs (Javaheri et al. 1997). The authors have already used omeprazole added to steroid therapy (prednisone) in order to alleviate the increased ICP and ameliorate the neurological status in a naturally ill dog with congenital hydrocephalus unresponsive to conventional medical therapy (Amude et al. 2013). However, the description about the usage of the omeprazole in order to treat a substantial group of affected dogs with suspected increased intracranial pressure by hydrocephalus is lacking. Accordingly, the aim of this paper was to present the results of a series of cases in which omeprazole was used for the medical management of hydrocephalus.

MATERIALS AND METHODS

This work revised the medical records of dogs with neurological deficits suggestive of prosencephalic dysfunction alone or in combination with other nervous signs in which the neurological diagnostic approach suggested hydrocephalus as presumptive diagnosis. All cases were assisted at the Veterinary Teaching Hospital from "Universidade de Cuiabá" (2011-2013) and medical follow up were performed by neurological service of the Hospital.

In all cases, hydrocephalus was diagnosed by clinical signs, neurological and physical exam, and ultrasonography of the brain (transcranial sonography - TCS) through open fontanelles and/or temporal window, as well as through foramen magnum window (Carvalho et al. 2007, Cintra et al. 2014). In addition to the TCS, in some cases, computed tomography (CT) was also performed to diagnose hydrocephalus and/or concomitant anomalous disease, when additional imaging was recommended to accomplish the final

diagnose. CT was carried out with a one channel helical tomography (Toshiba Xvision EX).

Hematological/biochemical evaluation, thoracic ray x, abdominal sonography, and cerebrospinal fluid (CSF) examination (lumbar puncture) were also performed in order to exclude asymptomatic subclinical hydrocephalus concomitant with systemic disease or other causes of encephalopathy as the cause of the neurological presentation. All animals were tested for canine distemper virus (CDV) antigen in CSF by rapid immunochromatographic test (Antigen Rapid CDV Ag Test Kit; Animal Genetics).

Omeprazole was used alone or in combination at the dose of 10mg/kg PO SID as previously described (Dewey 2008, Amude et al. 2013). When the animal was admitted to the Veterinary Teaching Hospital and no other medical management of hydrocephalus was previously started, the omeprazole was instituted as the unique drug in order to attempt medical treatment. When the animal was admitted with descompensated hydrocephalus in which conventional medical treatment (steroids and/or diuretics) had already being started previously, omeprazole was also added to the conventional medical treatment. The clinical response to medical treatment was scored subjectively as satisfactory (mild, moderate and optimal) or unsatisfactory.

The animals were followed up and transcranial sonography was performed through the period of clinical monitoring in order to assess the ventricular length during omeprazole therapy.

RESULTS

All dogs admitted by the neurological service though the period from 2011 to 2013 had the medical records revised, and 12 dogs were screened for this description since filled the criteria of the study; 6 dogs were diagnosed with non-obstructive hydrocephalus and in the other 6 cases hydrocephalus occurred with other concomitant anomalous encephalic disease often related with obstructive hydrocephalus (quadrigeminal cist - 1, arachnoid cyst - 1, chiary-like malformation - 3, and syringomyelia - 1). All of them had medical improvement after the use of omeprazole. In 10 dogs the omeprazole was used as single drug and showed optimal (n=8) to moderate clinical response (n=2). In 2 dogs medical treatment with steroids and/or diuretics had already been performed with mild to unsatisfactory medical control of the prosencephalic signs, however with the addition of omeprazole to the treatment they had improvement of the neurological status. The dog number, breed, neurological findings, diagnostic findings through imaginologic approach (TCS and/or CT), medical treatment (omeprazole singly or in addition with other drugs), response to therapy, ventricular size before and after omeprazole therapy, and time of clinical follow up were presented at Table 1. Six dogs had died during the follow up. Five died from non-neurological deterioration (respiratory failure, head trauma, acute not-known dead) and one dog died due to progressive neurological deterioration besides initial good response to the medical treatment.

The prosencephalic signs verified were characterized by circling, inappropriate consciousness content (behavioral change varying from fear to aggressiveness), compulsive walking, excessive vocalization, postural reactions deficits with normal to increased spinal reflex. Cranial nerves deficits were absent in the dogs with pure forebrain signs except bilateral absence of menace response (with normal fotomotor reflex). Divergent strabismus was also verified in

one instance. Bilateral divergent strabismus, a common sign of hydrocephalus, was only verified in one dog presented with the typical prosencephalic signs of the disease due to impairment of the third cranial nerve rather than cranium/orbita malformation due to hydrocephalus, since the strabismus was no more verified after one week of omeprazole therapy.

Rombencephalic signs (vestibular and/or cerebellar deficits) were verified in two instances concomitants to the prosencephalic signs; all of these dogs had hydrocephalus with concomitant neurological disease (quadrigeminal cist - 1, chiary-like malformation - 1). In two cases, rombencephalic signs (vestibular and cerebellar deficits) were the only nervous deficits, although hydrocephalus was recognized at imaginologic approach; such dogs were diagnosed with chiary-like malformation and concomitant hydrocephalus. Vestibular signs were characterized in these dogs by head tilt and/or positional strabismus; in addition, two dogs had rolling and tetraparesis. Cerebellar deficits were characterized by cerebellar ataxia (hypermetria with truncal and head ataxia) in one case, and hipermetria of the fore limb manifested during hopping test in the other three cases in which romboencephalic signs were recognized.

Hematological/biochemical evaluation, thoracic ray X, abdominal sonography, and CSF examination of all cases were within normal limits. Trauma was not suggested by the anamnesis, and the physical and laboratorial evaluations not showed any systemic abnormality. CDV antigen was not detected in CSF in any of the included dogs.

DISCUSSION

The importance of this report is the therapeutic use of omeprazole to ameliorate neurological signs in naturally affected dogs with hydrocephalus. Experimentally omeprazole was already proved to reduce ICP by decreasing the production of CSF in healthy rabbits (Lindvall-Axelsson et al. 1992) and dogs (Javaheri et al. 1997). The use of omeprazole for the treatment of hydrocephalus and syringomyelia in dogs has been suggested within the veterinary literature (Rusbridge et al. 2006, Dewey 2008, Thomas 2010, Estey 2016), and has being used by medical routine. The authors had already used omeprazole added to steroid therapy (prednisone) in order to ameliorate the neurological status in a naturally ill dog with congenital hydrocephalus unresponsive to conventional medical therapy (Amude et al. 2013). However, the use and effectiveness of omeprazole in clinical trials with naturally affected hydrocephalic dogs are lacking (Rusbridge et al. 2006, Thomas 2010, Estey 2016). To the best knowledge of the authors there are no description about the use of omeprazole in order to treat a substantial group of affected dogs with suspected increased intracranial pressure by hydrocephalus available within the scientific medical databases⁷.

Omeprazole, a specific inhibitor of H(+)-K(+)-activated ATPase (proton-pump inhibitor), has a dose-dependent inhibition of CSF production as determined by cerebroventriculocisternal perfusions in the rabbit; the reduction was 25% after an intravenous dose of 0.2 mg/kg of omeprazole (Lindvall-Axelsson et al. 1992). Initially the effect of omeprazole was thought to be due to the K(+)-H(+)-ATPase activity; however the effect

⁷ PubMed-NCBI <<http://www.ncbi.nlm.nih.gov/pubmed/>>, Scholar Google <<http://scholar.google.com.br/>>, SciELO <<http://www.scielo.org/php/index.php>>

Table 1. Distribution of the dogs, with final diagnosis, clinical data, ventricular measure, response to hydrocephalus treatment and time of clinical follow up

Dog	Age at presentation	Diagnosis	Clinical signs at the hospital presentation	Conventional medical treatment before omeprazole treatment	Degree of clinical response after conventional medical treatment	Length of the ventricles before omeprazole treatment (cm)	Length of the ventricles after omeprazole treatment (cm)	Degree of clinical response after omeprazole treatment	Time of clinical follow up
1 - Pinscher	50 days	Congenital hydrocephalus	Severe prosencephalic signs, blindness, bilateral ventrolateral strabismus	Corticosteroid (prednisone)	Mild response that deteriorate after 3 weeks	NA	NA	Satisfactory (moderate)	Six months (natural death)
2 - Shit-zu	2 years	Obstructive hydrocephalus and quadrigeminal cyst	Severe prosencephalic signs, central vestibular signs, tetraparesis and neuropathic pain (cervical)	NP	-	RLV-3.3 LLV-3.3 TV - NV	NP	Satisfactory (moderate)	Two week (death during immediate pos chirurgical to craniotomy and ventriculoperitoneal shunt)
3 - Pincher	6 months	Congenital hydrocephalus	Severe prosencephalic signs and neuropathic pain (cervical)	Corticosteroid (prednisone) Diuretic (furosemide)	Unsatisfactory	RLV-2.67 LLV-2.52 TV - NV	RLV-3.63 LLV-3.93 TV - NV	Satisfactory (mild)	One year (death with neurological deterioration due to progression of the hydrocephalus)
4 - Pinscher	5 months	Obstructive hydrocephalus and quadrigeminal cyst	Moderate prosencephalic signs and neuropathic pain (cervical)	NP	-	RLV - 1.1 LLV - 0.3 TV - NV	RLV - 0.6 LLV - 0.3 TV - NV	Satisfactory (optimal)	Seven months (died of cranial trauma unresponsive to medical treatment)
5 - Pinscher	2 years	Congenital hydrocephalus	Moderate prosencephalic signs and neuropathic pain (cervical)	NP	-	RLV-0.93 LLV-1.01 TV - 0.34x0.24	RLV - 0.6 LLV - 0.7 TV - 0.2x0.2	Satisfactory (optimal)	Two years and 3 months
6 - Poodle	Two month	Congenital hydrocephalus	Moderate prosencephalic signs	NP	-	NA	NA	Satisfactory (optimal)	Five years (died of respiratory failure because of tracheal collapse)
7 - Pinscher	54 days	Hydrocephalus and Chiari-like malformation and syringohidromielia	Moderate prosencephalic signs	NP	-	RLV - 1.2 LLV - 1.3 TV - NV	RLV - 0.9 LLV - 1.02 TV - NV	Satisfactory (optimal) during 3 months followed by Satisfactory (moderate)	Two years

Medical response: unsatisfactory or satisfactory (mild, moderate, optimal). Satisfactory: optimal = animal became clinical normal free of neurological signs, moderate = animal improved the neurological status, many of the nervous signs became absent but with persisted with some residual neurological signs, mild = animal improved the neurological status however persisted with all the signs previously registered at the beginning of the presentation; NA = not available, NP = not performed, NV= not verified, RLV = right lateral ventricle, LLV = left lateral ventricle, TV = third ventricle.

Table 1. Continued...

Dog	Age at presentation	Diagnosis	Clinical signs at the hospital presentation	Conventional medical treatment before omeprazole treatment	Degree of clinical response after conventional medical treatment	Length of the ventricles before omeprazole treatment (cm)	Length of the ventricles after omeprazole treatment (cm)	Degree of clinical response after omeprazole treatment	Time of clinical follow up
8 - Maltes	3 years	Hydrocephalus and Chiari-like malformation	Proencephalic signs, blindness, mild vestibular signs (head tilt and positional strabismus) and cerebellar signs (hipermetria of left forelimb)	NP	-	RLV - 0.9 LLV - 1.0 TV - 0.49x0.42	RLV - 0.63 LLV - 0.79 TV - 0.4x0.4	Satisfactory (optimal) for one moth followed by satisfactory (moderate) for four month and satisfactory (mild)	One year
9 - Yorkshire	20 days	Congenital hydrocephalus	Proencephalic signs, inadequate grow rate	NP	-	RLV-0.28 LLV-0.32 TV - NV	NP	Satisfactory (optimal) until acute repentine death	Two months (acute repentine death)
10 - Rottweiler	30 days	Congenital hydrocephalus	Proencephalic signs, inadequate grow rate	NP	-	RLV-0.72 LLV-0.59 TV - 0.26x0.3	NP	Satisfactory (optimal) until acute repentine death	One month (acute repentine death)
11 - Yorkshire	5 years	Hydrocephalus Chiari-like malformation	Vestibular signs (head tilt, positional strabismus, circling) and cerebellar signs (hipermetria of left forelimb)	NP	-	RLV-0.74 LLV-0.79 TV - 0.36x0.36	NP	Satisfactory (moderate)	Eight months
12 - Poodle	1 year and 7 months	Hydrocephalus Chiari-like malformation	Vestibular signs (head tilt, positional strabismus, circling/rolling)	NP	-	RLV - 1.0 LLV - 1.2 TV - NV	NP	Satisfactory (optimal)	One year and two months

Medical response: unsatisfactory or satisfactory (mild, moderate, optimal). Satisfactory: optimal = animal became clinical normal free of neurological signs, moderate = animal improved the neurological status, many of the nervous signs became absent but with persisted with some residual neurological signs, mild = animal improved the neurological status however persisted with all the signs previously registered at the beginning of the presentation; NA = not available, NP = not performed, NV = not verified, RLV = right lateral ventricle, LLV = left lateral ventricle, TV = third ventricle.

is independent of the K(+)-H(+)-ATPase activity, since Sch 28080 - a potent inhibitor of H(+)-K(+)-ATPase - which is more potent than omeprazole did not significantly affect CSF production (Javaheri et al. 1997). In addition to the studies with rabbits, experimental studies in canine model also suggested that omeprazole may decrease CSF production by about 26% (Javaheri et al. 1997).

These previous studies were carried out with healthy animals without increased ICP, and the extrapolation of those preliminary results for clinical usage of omeprazole in situations where the limited CSF production should be beneficial, such as hydrocephalus, might be done with caution. In order to check this, the application of omeprazole should be tested in ill dogs in which the limitation of CSF could ameliorate the signs of the disease, such as the 12 hydrocephalic dogs presented herein. The present description shown that the animals had not only clinical improvement, as well had reduction of the ventricular size.

Girod et al. (2016) suggested that CSF production in healthy dogs may not be affected by chronic oral therapy with omeprazole at the dose of 1.2mg/kg, differing from the previous results where administration of omeprazole by ventriculo-cisternal perfusion or intravenously shown to decrease CSF production in healthy dogs and rabbits (Lindvall-Axelsson et al. 1992, Javaheri et al. 1997). The difference of these results might be in part due to the administration route or the method employed to quantify the decrease of CSF production; while Lindvall-Axelsson et al. (1992) and Javaheri et al. (1997) had quantified by open system and canulation of CSF flow, Girod et al. (2016) had measured by albumin quotient determination in CSF samples. However, in the presented clinical description, at the dose of 10mg/kg, oral omeprazole could improve the neurological status of the hydrocephalic dogs, probable due to limiting the CSF production, since the most of the cases had ventricular size reduction after omeprazole therapy. Prospective studies in naturally affected dogs have been carried out by the authors in order to clarify the therapeutic use of omeprazole in other diseases that naturally course with increased ICP such as tumors and head trauma.

CONCLUSIONS

The results of this study shown that in naturally affected dogs with hydrocephalus, omeprazole may be used orally at the dose of 10mg/kg in order to and ameliorate the neurological status. Furthermore, the results suggested that omeprazole can be used in cases other than only congenital non-obstructive hydrocephalus including quadrigeminal cist, arachnoid cyst, chiary-like malformation, and syringomyelia, even when affected dogs are presented with only rombocephalic signs without prosencephalic deficits.

This work may represent the first clinical description using omeprazole in order to treat a substantial group of affected dogs with suspected increased intracranial pressure by hydrocephalus.

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Ganciclovir attenuates the respiratory disease induced by *Equid alphaherpesvirus 1* in rabbits¹

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ABSTRACT.- Mortari A.P.G., Amaral B.P., De Oliveira P.S.B., Dotto E.K., Flores M.M., Cargnelutti J.F., Weiblen R. & Flores E.F. 2019. **Ganciclovir attenuates the respiratory disease induced by *Equid alphaherpesvirus 1* in rabbits.** *Pesquisa Veterinária Brasileira* 39(10):830-836. Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Av. Roraima 1000, Rua Z, Prédio 63A, Santa Maria, RS 97105-900, Brazil. E-mail: eduardofurtadoflores@gmail.com

Equid alphaherpesvirus 1 (EHV-1) is an important pathogen of horses, associated with respiratory, neurological disease and abortions. As vaccination is not always effective, anti-herpetic therapy may represent an alternative to prevent the losses caused by the infection. We herein investigated the activity of ganciclovir (GCV), an anti-herpetic human drug, in rabbits experimentally infected with EHV-1. Thirty-days-old New Zealand rabbits were allocated in three groups (6 animals each) and submitted to different treatments: G1 (non-infected controls), G2 (inoculated with EHV-1) - 10^7 TCID₅₀ intranasally - IN) and G3 (inoculated IN with EHV-1 and treated with GCV - 5mg/kg/day for 7 days) and monitored thereafter. All animals of G2 developed systemic signs (moderate to severe apathy, anorexia), ocular discharge and respiratory signs (serous to mucopurulent nasal discharge), including mild to severe respiratory distress. Viremia was detected in all rabbits of G2 for up to 11 days (mean duration = 6.5 days). One animal died after severe respiratory distress and neurological signs (bruxism, opisthotonus). In addition, these animals gained less weight than the control (G1) and GCV-treated rabbits (G3) from days 4 to 14pi ($p < 0.05$). The clinical score of rabbits of G2 was statistically higher than the other groups from days 3 to 6pi ($p < 0.05$), demonstrating a more severe disease. In contrast, G3 rabbits did not present systemic signs, presented only a mild and transient nasal secretion and gained more weight than G2 animals ($p < 0.05$). In addition, viremia was detected in only 3 rabbits and was transient (average of 2.3 days). Thus, administration of GCV to rabbits inoculated IN with EHV-1 resulted in an important attenuation of the clinical disease as demonstrated by full prevention of systemic signs, maintenance of weight gain and by drastic reduction in viremia and in the magnitude of respiratory signs. These results are promising towards further testing of GCV as a potential drug for anti-herpetic therapy in horses.

INDEX TERMS: Ganciclovir, respiratory disease, equid alphaherpesvirus 1, rabbits, herpesvirus, therapy, EHV-1, animal model.

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RESUMO.- [O ganciclovir atenua a doença respiratória produzida pelo alfavírus equino 1 em coelhos.]

O alfavírus equino 1 (EHV-1) é um importante patógeno de equinos, associado com doença respiratória, neurológica e abortos. Como a vacinação nem sempre é eficaz, a terapia anti-herpética pode representar uma alternativa para prevenir as perdas causadas pela infecção. Para tal, investigou-se a atividade do ganciclovir (GCV), uma droga anti-herpética de uso humano, em coelhos infectados experimentalmente com o EHV-1. Coelhos da raça Nova Zelândia com 30 dias de idade foram alocados em três grupos (6 animais cada) e submetidos

a diferentes tratamentos: G1 (controles não infectados), G2 (inoculados com o EHV-1) - 10^7 TCID₅₀ intranasal - IN) e G3 (inoculados IN com o EHV-1 e tratados com GCV - 5mg/kg/dia por 7 dias), e monitorados posteriormente. Todos os animais do G2 desenvolveram sinais sistêmicos (apatia moderada a grave, anorexia), secreção ocular e sinais respiratórios (secreção nasal serosa a mucopurulenta), incluindo dificuldade respiratória leve a grave. Viremia foi detectada em todos os coelhos do G2 por até 11 dias (duração média = 6,5 dias). Um animal morreu após dificuldade respiratória grave e sinais neurológicos (bruxismo, opistótono). Além disso, esses animais ganharam menos peso que os coelhos controle (G1) e tratados com GCV (G3) entre os dias 4 e 14pi ($p < 0,05$). O escore clínico de coelhos do G2 foi estatisticamente maior que os demais grupos dos dias 3 a 6pi ($p < 0,05$), demonstrando uma doença mais grave. Em contraste, os coelhos do G3 não apresentaram sinais sistêmicos, apresentaram apenas secreção nasal leve e transiente e ganharam mais peso que os animais do G2 ($p < 0,05$). Além disso, a viremia foi detectada em apenas 3 coelhos e foi transitória (média de 2,3 dias). Assim, a administração de GCV a coelhos inoculados com EHV-1 resultou em uma importante atenuação da doença clínica, como demonstrado pela prevenção completa de sinais sistêmicos, manutenção do ganho de peso e pela redução drástica da viremia e da magnitude dos sinais respiratórios. Estes resultados são promissores para testes adicionais com o GCV para potencial terapêutico anti-herpético em equinos.

TERMOS DE INDEXAÇÃO: Ganciclovir, doença respiratória, alphaherpesvírus equino 1, *Equid alphaherpesvirus 1*, coelhos, herpesvírus, terapia, EHV-1, modelo animal.

INTRODUCTION

Equid alphaherpesvirus 1 (EHV-1) is an enveloped, double-stranded DNA virus belonging to the genus *Varicellovirus*, subfamily Alphaherpesvirinae, family Herpesviridae (ICTV 2018). EHV-1 infection is distributed worldwide and is frequently associated with respiratory disease, abortion, perinatal foal mortality and/or neurological disease (equine herpesvirus myeloencephalopathy - EHM) (Allen & Bryans 1986, Allen & Umphenour 2004, Lunn et al. 2009, Goodman et al. 2012, Ma et al. 2013, Pusterla & Hussey 2014). After acute infection, EHV-1 establishes lifelong latent infections, mainly in sensory nerve ganglia but also in lymphoid cells (Welch et al. 1992, Pusterla et al. 2010). Natural reactivation of latent infection results in virus replication and transmission to susceptible animals, representing a means of virus perpetuation in nature (Patel & Heldens 2005).

The respiratory disease associated with EHV-1 infection is especially important in young racing horses submitted to intensive training since it affects the physical performance (Gilkerson et al. 1999). The reproductive losses by abortions, perinatal foal mortality and neurological disease (EHM) resulting in high mortality, in addition to the restrictions to transportation, contribute to the economic impact of EHV-1 infection (Lunn et al. 2009).

Although vaccines against EHV-1 are available, they are not fully protective such as outbreaks of disease have been reported even in regularly vaccinated herds (Allen & Umphenour 2004, Kydd et al. 2012). Likewise, no specific treatment is available for the disease caused by EHV-1. Then, control measures such

as support therapy and animal isolation have been indicated to reduce transmission and to minimize clinical signs (Carmichael & Greene 2006). Considering the high economic value of some horses, EHV-1 infection represents an attractive target for testing and, eventually employing antiviral drugs (Schwers et al. 1980, Maggs & Clarke 2004, Bentz et al. 2006, Marley et al. 2006, Meulen et al. 2006, Garré et al. 2007a).

A number of drugs have been successfully used for the treatment of herpetic infections in humans, yet their use in veterinary medicine is still incipient (De Clercq 2008, Razonable 2011, Maxwell 2017). However, some studies investigated the antiviral action of drugs against bovine herpesviruses (1, 2 and 5) (Dezengrini et al. 2010), felid herpesvirus (Thomasy & Maggs 2016) and herpesviruses of horses (Vissani et al. 2015, Oliveira et al. 2018). These drugs may have important applications in equine medicine by reducing the clinical consequences of herpetic infections (Garré et al. 2008, Carmichael et al. 2013, Glorieux et al. 2012, Maxwell et al. 2008, Maxwell 2017).

Antiviral drugs include the nucleoside analogs acyclovir (ACV), ganciclovir (GCV), cidofovir (CDV), famciclovir (FAM), foscarnet (FOS) and vidarabine (VID) (Coen & Richman 2007). The action of these drugs occurs via their incorporation into the nascent DNA molecule, halting the DNA polymerase from completing polymerization (Whitley & Roizman 2001, Coen & Richman 2007, Levinson 2016). A number of studies *in vitro* has indicated the efficacy and safety of some of these drugs against EHV-1, especially GCV (Vissani et al. 2015, Oliveira et al. 2018), which was the most effective and safest drug among six drugs tested *in vitro* (Oliveira et al. 2018). Furthermore, the pharmacokinetics of GCV in horses has been already studied (Carmichael et al. 2013). The activity of GCV in horses with meningoencephalitis by EHV-1 has been demonstrated (Maxwell 2017), yet its antiviral activity in the respiratory disease induced by EHV-1 has not been investigated.

GCV was the first antiviral drug used for the treatment of cytomegalovirus (CMV) infections, and currently it is widely used in immunosuppressed patients (HIV infected, transplanted, etc.) that develop secondary CMV infection. GCV is an acyclic analog of the nucleoside guanosine, and when at triphosphate state, it is a competitive inhibitor of deoxyguanosine triphosphate incorporation into DNA and inhibits viral DNA polymerases, serving as a poor substrate for chain elongation, thereby disrupting viral DNA synthesis (Crumpacker 1996).

Thus, in this article we describe an investigation of the antiviral activity of GCV in a rabbit model for EHV-1 respiratory disease. Our results are promising towards further testing of GCV in herpetic infections of horses.

MATERIALS AND METHODS

The activity of GCV against EHV-1 *in vivo* was investigated in rabbits inoculated intranasally (IN) with strain Kentucky D. All procedures involving animals were performed under veterinary supervision and conducted following the guidelines by the Brazilian Committee of Animal Experimentation (COBEA, law no. 6,638, of May 8th, 1979). The animal experiment was approved by the Institutional Committee of Ethic and Animal Experimentation Welfare (UFMS - "Comitê de Ética e Experimentação Animal": CEUA no. 3364110719).

Cells and virus. Vero cells (African Green Monkey kidney) were used in all procedures of virus amplification, quantitation, isolation

from clinical specimens and virus-neutralizing (VN) assays. Cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (Cultilab, Inc.), added of antibiotics (streptomycin 0.4mg/mL; penicillin 1.6mg/mL) and antifungic (anphotericin B 0.0025mg/mL). The EHV-1 Kentucky D strain was kindly provided by Dr Rodrigo Franco (Instituto Butantan, São Paulo/SP, Brazil).

Animals and virus inoculation. Eighteen New Zealand rabbits (approximately 30-days-old) were allocated in three groups of six animals each according to the respective treatments: G1 (uninfected controls, inoculated IN with RPMI medium); G2 (inoculated with EHV-1); G3 (inoculated with EHV-1 and treated with GCV). Animals from G2 and G3 were inoculated into the paranasal sinuses (Kanitz et al. 2015) with 1mL of culture supernatant containing 10^7 mean tissue culture infectious doses (TCID₅₀) of EHV-1 after sedation with ketamine (50mg/kg) and xylazine (5mg/kg). Starting at the day of virus inoculation, animals of G3 received GCV intravenously (5mg/kg/day) during seven days.

Animal monitoring and sample testing. Experimental animals were weighted and monitored in a daily basis for clinical and virological aspects up to day 15 post-inoculation (pi). Clinical monitoring involved a close observation of behavior, food ingestion, alertness, ocular and respiratory signs. A clinical score was determined per group/day by the sum of the values of the following parameters: apathy (presence: 1, absence: 0); ocular secretion (presence: 1, absence: 0), nasal discharge (serous: 1, mucopurulent: 2, absence: 0), respiratory distress (mild: 1, mild to moderate: 2, moderate to severe: 3, absence: 0), neurological signs (presence: 1, absence: 0), death (yes: 1, no: 0).

Nasal swabs were collected in alternate days up to day 15pi and submitted to virus isolation. For virus isolation, swabs collected in 0.5mL of RPMI medium were drained, centrifuged (1,000xg for 10min) and the 0.1mL of the supernatant was inoculated onto Vero cell monolayers grown in 24-well plates. Samples were submitted to three passages of four days each and monitored for cytopathic effect (cpe). Lung fragments collected from rabbit #28 (G3) which died at day 3pi were also submitted to virus isolation in Vero cells.

Viremia was investigated by submitting blood collected in alternate days (50µl/animal) to DNA extraction by phenol-chloroform and a PCR for EHV-1 gB gene according to Kanitz et al. (2015). Total DNA extracted from uninfected Vero cells and from cells inoculated with EHV-1 was used as negative and positive controls, respectively.

Blood for serology was collected at days 0 and 15pi. Serum samples were submitted to a standard microtiter virus-neutralizing (VN) assay, testing two-fold dilutions of serum against a fixed dose of virus (100-200 TCID₅₀). Vero cells were used as indicators and the readings were performed at 96h of incubation. VN titers were considered the reciprocal of the highest serum dilution capable of preventing the production of cpe.

Lungs collected from rabbit # 28 were fixed in 10% buffered formalin, embedded in paraffin, cut at 5µm and stained with hematoxylin and eosin for microscopic examination.

Statistical analysis. The weight values and clinical score obtained daily from all experimental animals were submitted to variance analysis by the Two-way ANOVA method, with Tukey test for multiple comparisons, using the GraphPad Prism (version 6) software. Differences between the groups were considered significant when the *p* values were lower than 5% (*p*<0.05).

RESULTS

All six rabbits inoculated with EHV-1 (G2) developed systemic and respiratory signs from day 2 to 11pi, shed virus in nasal secretions from day 1 to 13pi and seroconverted to EHV-1 (VN titers from 8 to 128) (Table 1). The disease was characterized by moderate to severe apathy and anorexia, serous ocular secretion, nasal discharge (serous to muco-purulent) and mild to severe respiratory distress. Rabbit # 28 died at day 3pi after a course of respiratory distress, bruxism and opisthotonus. Infectious virus and histological changes compatible with virus-associated inflammation were observed in the lungs of this animal. Viremia was detected in G2 animals from day 3 to 13pi (mean duration = 6.5 days). In addition, G2 rabbits presented a reduced weight gain comparing to uninfected controls (G1) and GCV treated rabbits (G3) from day 4 to 14pi (*p*<0.05). These results demonstrated the production of systemic and respiratory disease by EHV-1 in rabbits infected experimentally.

In contrast, no systemic signs (changes in alertness, appetite) or overt respiratory signs were observed in rabbits inoculated with EHV-1 and receiving GCV treatment (G3). The clinical score, compiling respiratory and systemic signs, was statistically higher (*p*<0.01) in G2 rabbits than in GCV treated animals (G3) from day 3 to 6pi (Fig.1), illustrating

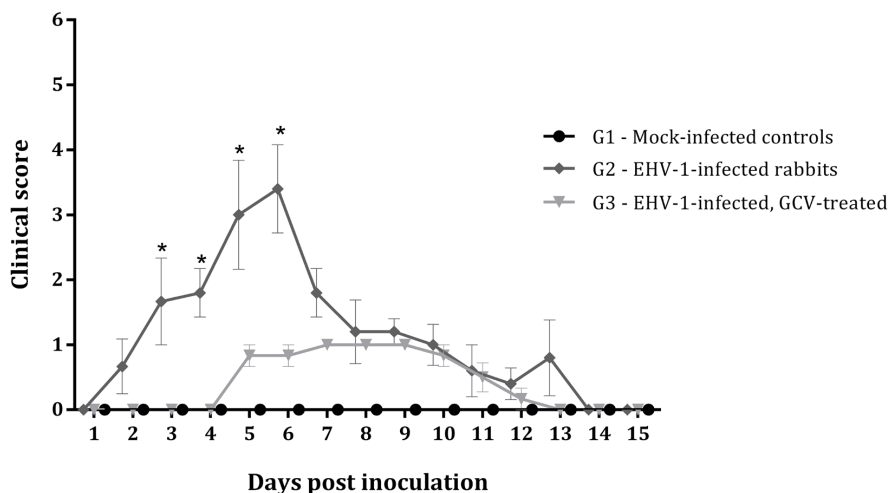


Fig.1. Mean of clinical scores obtained daily from the groups of rabbits experimentally infected with EHV-1 (G2) and infected with EHV-1 and treated with ganciclovir (G3). Statistical difference between groups G2 and G3 (*), *p*<0.001.

the important reduction in the magnitude of clinical signs in animals receiving GCV (G3). These animals presented only a mild serous nasal secretion between days 5 and 12pi. The weight gain of these animals was comparable to the

uninfected control group and higher than the G2 group at days 4, 6, 10, 12 and 14 ($p < 0.05$) (Fig.2). G3 animals shed virus in nasal secretions from day 1 to 15pi and 5/6 seroconverted to EHV-1 at day 15pi, harboring VN titers from 8 to 32 (Table 1).

Table 1. Clinical, virological and serological findings of equid alphaherpesvirus 1 (EHV-1) infected rabbits and treated with ganciclovir (GCV)

Animal #	Viral shedding (dpi ^a)	Viremia (dpi)	Clinical signs (dpi)	Neutralizing antibodies 15dpi
G1 - mock-infected controls				
8	- ^b	-	Healthy	< 2
9	-	-	Healthy	< 2
10	-	-	Healthy	< 2
11	-	-	Healthy	< 2
12	-	-	Healthy	< 2
13	-	-	Healthy	< 2
G2 - EHV-1 infected				
26	3	3-13	Apathy, serous and mucopurulent nasal discharge, ocular secretion, mild respiratory distress (3-11)	8
27	1-5	5-13	Apathy, serous and mucopurulent ocular and nasal discharge, mild to moderate respiratory distress (3-10)	8
28	1-3*	3	Apathy, serous nasal discharge, severe respiratory distress, bruxism, opisthoton, death (3 dpi)* (2-3)	n.p.
29	5-11	3-13	Apathy, serous and mucopurulent nasal discharge, mild respiratory distress (3-10)	128
30	1-13	5-9	Apathy, serous and mucopurulent nasal discharge (3-9)	16
31	1**	13	Apathy, serous and mucopurulent nasal discharge, mild to moderate respiratory distress (3-7)	n.p.
G3 - EHV-1 infected and treated with GCV				
20	3-5	-	Mild serous nasal secretion (5-10)	32
21	1-11	-	Mild serous nasal secretion (5-11)	32
22	1-9	-	Mild serous nasal secretion (5-9)	8
23	7-15	7	Mild serous nasal secretion (5-11)	16
24	1-15	3-7	Mild serous nasal secretion (5-12)	<2
25	1-11	7	Mild serous nasal secretion (7-10)	32

^a Days post-inoculation, ^b not-detected, * dead at day 3pi, ** dead at day 14pi; n.p. = not performed.

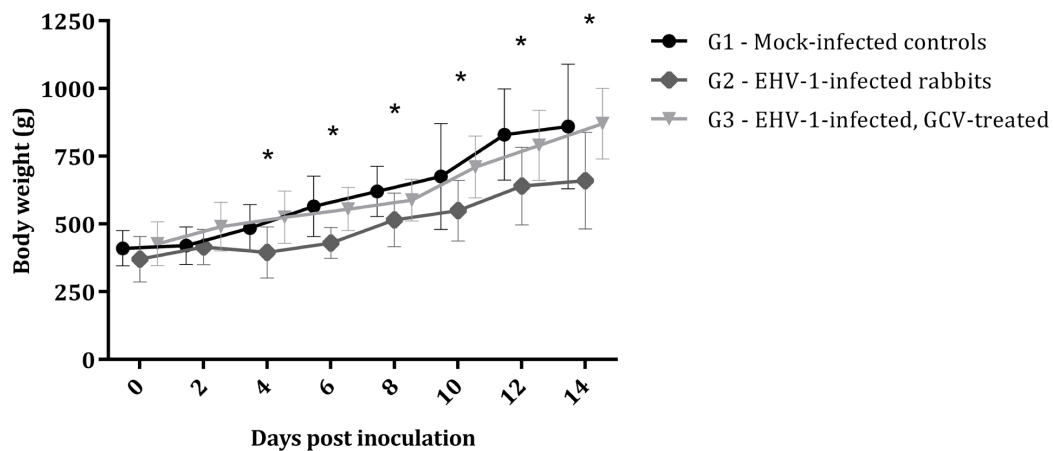


Fig.2. Mean and standard deviation of the mean of body weight of control group (G1), EHV-1 inoculated rabbits (G2) and EHV-1 inoculated rabbits and treated with ganciclovir (G3). Significant statistical difference between groups (*), $p < 0.05$. No statistical differences in weight gain was between G1 and G3.

Transient viremia was detected in only 3 rabbits and lasted an average of 2.3 days. These results demonstrate that GCV treatment fully prevented the development of systemic signs, preserved weight gain, reduced viremia and resulted in an important attenuation of the systemic and respiratory disease.

No difference in virus shedding (duration) was evident between rabbits from G2 and G3 (Table 1). Uninfected rabbits remained healthy, did not shed virus or presented viremia, gained weight and remained seronegative to EHV-1 throughout the experiment.

DISCUSSION

The administration of GCV to rabbits inoculated IN with EHV-1 resulted in an important attenuation of the clinical disease as demonstrated by full prevention of systemic signs, maintenance of weight gain and by a drastic reduction in viremia and in the magnitude of respiratory signs. As one animal from G2 (# 28) died after severe respiratory and neurological signs, the prevention of mortality may be also attributed to GCV treatment. These results may pave the way for further studies in rabbits and, eventually in horses, to investigate the real potential of GCV as an anti-herpetic drug for equine medicine. Although antiviral treatment of animals may be expensive due to difficulties to obtain GCV in large scale, the benefits of treating horses against EHV-1 infection may be rewarding.

Anti-herpetic therapy has long been used in human medicine yet its application in veterinary medicine is still incipient. Since vaccination is not always effective in preventing respiratory, neurological disease and abortions associated with EHV-1 infections, antiviral therapy may represent an attractive alternative to reduce the losses caused by these conditions. In fact, human anti-herpetic drugs have been tested for the therapy of diseases caused by EHV-1 and other equine herpesviruses as well (reviewed by Vissani et al. 2015, Maxwell 2017). However, many evidences of anti-herpetic drug efficacy against equine herpesviruses are derived from studies *in vitro* or in animal models, from empirical data, with limited testing and/or clinical experience in horses (Maxwell 2017). In fact, although investigation of anti-viral therapy has been performed for several equine herpesviruses, only diseases associated with EHV-1 and EHV-5 infections have been treated with anti-herpetic drugs in routine equine practice (Vissani et al. 2015, Maxwell 2017).

Acyclovir and its prodrug, valacyclovir are the human anti-herpetic drugs more commonly tested in EHV therapy. Oral valacyclovir has been used to protect adult horses from EHV-1 myeloencephalopathy. Oral acyclovir is frequently administered for the therapy of equine multinodular pulmonary fibrosis associated with EHV-5 infection. Other anti-herpetic drugs are promising but require further investigation (reviewed by Maxwell 2017).

GCV has been the drug of choice to replace acyclovir (ACV) in the therapy of herpes simplex virus infections in humans since it is less toxic and more stable (Shen et al. 2009). The efficacy of GCV in attenuating clinical disease has been demonstrated for different human herpesviruses, including Epstein-Barr virus (Egan et al. 2011), CMV in immunodepressed patients (Lalezari et al. 2002), keratitis by HSV-1 (Wang et al. 2015) and encephalitis by HHV-6 (Mookerjee & Vogelsang 1997).

Several studies have shown that GCV is, at least, 10 times more potent than acyclovir and penciclovir against EHV-1 *in vitro* and in mice (reviewed by Maxwell 2017, Oliveira et al. 2018). In addition, GCV presents a favorable pharmacokinetic profile and it is well tolerated for horses (Carmichael et al. 2013). Investigation of GCV anti-EHV-1 activity in cases of neurological disease, however, has yielded conflicting results. GCV administration to experimentally infected horses at the onset of fever was able to reduce the severity of neurological signs, viremia and temperature (Maxwell 2017). On the other hand, GCV treatment of two naturally infected horses developing mioencephalopathy was unable to reduce the severity of clinical disease and death (Estell et al. 2015). The activity of GCV against EHV-1 *in vitro* has been demonstrated, being a potent and selective compound (Oliveira et al. 2018). Regardless, the activity of GCV in the respiratory disease by EHV-1 remained unexploited. In this study we investigated its activity *in vivo*, using a rabbit model for EHV-1 respiratory disease established previously (Kanitz et al. 2015).

Treatment of rabbits inoculated with EHV-1 with GCV starting at the day of virus inoculation was effective in preventing systemic disease, in maintaining weight gain and drastically reducing viremia and the severity of respiratory signs. Interestingly, GCV treatment apparently did not interfere with virus replication in the nasal mucosa since the duration of virus shedding through nasal secretions was similar in G2 and G3 groups. It is conceivable that GCV may have interfered with virus replication in the lower respiratory tract, especially in the lungs, what would explain the prevention of systemic disease and attenuation of respiratory signs observed in treated animals (G3). Demonstration of infectious virus and inflammatory changes in the lungs of rabbit #28, which died after severe respiratory and neurological signs, reinforce that EHV-1 pathogenesis involves replication in lower respiratory tract. Indeed, pulmonary inflammation secondary to EHV-1 infection can cause substantial morbidity and mortality in neonatal foals and upper respiratory signs in weanlings (Murray et al. 1998, Brown et al. 2007). Drastic reduction in viremia reinforces that GCV treatment, at some stage, reduced EHV-1 replication and/or systemic spread.

The therapeutic protocol, including drug dose, route and interval of administration and duration was adapted from previous studies of pharmacokinetics and toxicity in rabbits and horses (Hedaya & Sawchuk 1990, Carmichael et al. 2013) and based on previous studies showing antiviral activity *in vitro* (Garré et al. 2007a, Oliveira et al. 2018). Implementing GCV administration at the day of virus inoculation, however, is rather unusual and obviously does not reflect the clinical practice. By doing so, we tried to proof the concept of GCV activity *in vivo* against EHV-1-induced respiratory disease. Whether GCV will be capable of attenuating clinical disease by EHV-1 when administered after the onset of clinical signs will demand further studies. In any case, our results demonstrated anti-herpetic activity of GCV *in vivo* and are, therefore, promising towards further studies to define the real potential of GCV as a drug for therapy of herpetic infections in horses.

In any case, our results are promising towards the use of GCV in the therapy of EHV-1 disease in horses and open the way for further studies of GCV activity against EHV-1 respiratory disease in animal models and, in a second phase, in its natural host.

CONCLUSION

Intravenous administration of GCV at a dose of 2.5mg/kg every 12h for 7 days in rabbits inoculated with EHV-1 by the intranasal route was effective in preventing systemic signs, attenuating respiratory disease, reducing viremia and preserving weight gain, when comparing with untreated rabbits. These results indicate that this drug may be a good alternative for experimental studies in horses, aiming to establish an anti-herpetic therapeutic protocol for this species.

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Behaviour of different types of chitosan membranes implanted in horses¹

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ABSTRACT. Rollim V.M., Reginato G.M., Fernandes L.M., Arantes J.A., Rigo E.C.S., Vercik L.C.O., Freitas S.H. & Dória R.G.S. 2019. **Behaviour of different types of chitosan membranes implanted in horses.** *Pesquisa Veterinária Brasileira* 39(10):837-842. Departamento de Medicina Veterinária, Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Campus Fernando Costa, R. Duque de Caxias 225, Jardim Elite, Pirassununga, SP 13635-900, Brazil. E-mail: redoria@usp.br

Chitosan has been successfully used as a biomaterial with several purposes in many species. In this study, chitosan membranes were produced with six different types of materials, and their behavior were evaluated upon implantation in the subcutaneous tissue of the flank of twelve healthy horses. We assessed chitosan membranes obtained from commercial chitosan, impregnated or not with silver nanoparticles, sterilized with ethylene oxide (CCEO, n=3; CCSNEO, n=3) or by ultraviolet radiation (CCUR, n=3; CCSNUR, n=3), and chitosan membranes obtained from squid gladius, sterilized with ethylene oxide (SCEO, n=6) or by ultraviolet radiation (SCUR, n=6). The same animals were randomly used in two experimental groups, with a minimum interval of 60 days between procedures, respecting the fact of only one flank side, left or right, be under evaluation by experimental period. After preparation of the membranes and implantation in the flank subcutaneous tissue of the horses, macroscopic and ultrasonographic evaluations of the implant regions were performed, as well as physical examination, blood count and fibrinogen measurement. No clinical or laboratory abnormalities were observed. All animals that received commercial chitosan membranes, regardless of the preparation technique, showed rejection to the biomaterials, considering that 100% of the surgical wounds presented dehiscence of suture and expulsion of the implants. The animals that received squid gladius chitosan membranes showed success in the treatment, with healing by primary intention of the surgical wound. We conclude that squid gladius chitosan membranes are biocompatible and biodegradable when implanted in the subcutaneous tissue of the flank of healthy horses.

INDEX TERMS: Chitosan membranes, horses, implant, biomaterial, wound healing.

RESUMO.- [Comportamento de diferentes tipos de membranas de quitosana implantadas em equinos.]

A quitosana tem sido utilizada, com sucesso, como biomaterial para diversas espécies e finalidades. Neste estudo foi avaliada

a confecção de membranas de quitosana, produzidas a partir de seis tipos de materiais diferentes e foi estudado seu comportamento quando implantadas no tecido subcutâneo do flanco de doze equinos sadios. Foram avaliadas membranas de quitosana obtidas de quitosana comercial, impregnadas ou não com nanopartículas de prata, esterilizadas com óxido de etileno (QCOE, n=3; QCNPOE, n=3) ou por radiação ultravioleta (QCRU, n=3; QCNPUR, n=3) e membranas de quitosana obtidas do gládio de lula, esterilizadas com óxido de etileno (GLOE, n=6) ou por radiação ultravioleta (GLRU, n=6). Os mesmos animais foram utilizados em dois grupos experimentais, de forma aleatória, com um intervalo mínimo de sessenta dias entre os procedimentos, respeitando-se o fato de apenas um lado

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do flanco, esquerdo ou direito, estar em avaliação por período experimental. Após preparo das membranas e implantação no tecido subcutâneo do flanco dos equinos, foram realizadas avaliações macroscópicas e ultrassonográficas das regiões de implante, além de exames físicos, hemogramas e fibrinogênio. Não foram observadas alterações clínicas e laboratoriais. Todos os animais que receberam membranas de quitosana comercial, independente da técnica de preparo, demonstraram rejeição dos biomateriais, uma vez que 100% das feridas cirúrgicas apresentaram deiscência da sutura e expulsão dos implantes. Os animais que receberam as membranas de quitosana de gládio de lula demonstraram sucesso no tratamento, com cicatrização das feridas cirúrgicas por primeira intenção. Conclui-se que membranas de quitosana de gládio de lula são biocompatíveis e biodegradáveis, quando implantadas no tecido subcutâneo do flanco de equinos saudáveis.

TERMOS DE INDEXAÇÃO: Membranas de quitosana, equinos, implante, biomaterial, cicatrização feridas, quitosana.

INTRODUCTION

Biomaterials of biological or synthetic nature can be widely used in the medical field as long as they are biocompatible, i.e., non-toxic, non-carcinogenic and non-antigenic, and do not stimulate inflammatory processes or favor infections (Alexander 1987, Iamaguti et al. 1995, Melo et al. 1998, Akamoto & Trento 2002, Alievi et al. 2007, Turrer & Ferreira 2008, Raposo-do-Amaral et al. 2010, Kuhl et al. 2017).

Chitosan, a chitin-derived polysaccharide, is the major component of the exoskeleton of crustaceans, mollusks and insects and of the cell wall of fungi and yeast. It has been used as biomaterial because of its numerous properties, such as biocompatibility and bioadhesiveness and action in the opening of epithelial tight junctions (Kumar 2000, 2004, Berger et al. 2004). Chitosan presents antimicrobial action, homeostatic activity, ability to activate macrophages, stimulate cell migration and proliferation, promote granulation tissue formation, and guide the reorganization of cellular histoarchitecture in wounds (Muzzarelli 2009, Kuhl et al. 2017). One of the most promising characteristics of chitosan for use as biomaterial is its excellent ability to be processed into porous structures, such as membranes, for use in cell transplantation, dressings and drug delivery (Denkbass & Ottenbrite 2006).

The use of chitosan for wound healing as dressings developed in the form of sponges and porous membranes has been proven quite effective (Brown et al. 2009). In animal model experiments, chitosan has been shown to have a positive influence on all stages of tissue healing (Alemdaroglu et al. 2006, Muzzarelli 2009). In horses, incisional infection is a concern in the postoperative period, as it delays surgical wound healing, resulting in increased postoperative care and convalescence, as well as in the need for surgical reintervention, as in cases of incisional hernias (Freeman 2018). In this interval, it is advantageous to apply measures that reduce suppuration of the surgical wound in horses, such as the use of chitosan membranes, impregnated or not with antimicrobials, implanted in the subcutaneous tissue, in order to favor healing of the contact tissues such as muscles and skin.

One of the drawbacks of using implants in horses, whether of animal or synthetic origin, is the tissue reaction, or even rejection, that this species presents against tissues considered

foreign to the body (Whitfield-Cargile et al. 2011). To date, no studies have been found in the literature addressing chitosan implantation in equine surgical wounds. Thus, this study aimed to develop different protocols for chitosan membrane production and evaluate their behavior when implanted in the subcutaneous flank tissue of healthy horses.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of the aforementioned Institution under protocol no. 9840070118.

Preparation of commercial chitosan membranes. Chitosan membranes were prepared using the casting technique. A 2% (w/v) commercial chitosan solution (Sigma-Aldrich®, low molecular weight) solubilized in 1% acetic acid solution (v/v) was prepared. After that, 25mL of the solution were poured in a 90x15mm Petri dish. The Petri dishes were then stored in a forced air circulation oven (Rainbow®) for 48h.

Preparation of commercial chitosan membranes impregnated with silver nanoparticles. The silver nanoparticles (AgNPs) were obtained using a commercial chitosan 6.92mg/mL solution (Sigma-Aldrich®, low molecular weight) diluted in 1% acetic acid (v/v) and a silver nitrate solution (Tec-Lab®) with concentration of 12mM diluted in distilled water. Both solutions were mixed and homogenized using a TE-0851 magnetic stirrer (Tecnal®) for approximately 15min, and then placed in a water bath at 90°C for 18h (Wei & Qian 2008).

The membranes were obtained from the AgNPs solution, with concentration adjustment up to 2% (m/v). Then 25mL of the solution were poured in a 90x15mm Petri dish and the membranes were dried in a forced air circulation oven (Rainbow®) for 48h.

Preparation of commercial chitosan membranes from squid gladius. The chitosan used in this stage was obtained from the extraction of β -chitin from pens of the squid species *Doryteuthis* spp. according to established methodology.

Squid pens provided by the Research Group in Biochemistry and Biomaterials of the "Instituto de Química de São Carlos" (IQSC-USP) were washed in running water to remove salts and organic material, oven dried at 40°C, crushed, and separated using 0.250mm sieve. Extraction of β -chitin from the squid pens and the preparation of chitosan were performed by deproteinization and N-deacetylation, respectively, adapting a method described in the literature (Horn et al. 2009).

A 2% chitosan (m/v) 1% acetic acid (v/v) solution was prepared. This mixture was kept under constant stirring at room temperature until complete solubilization. After that, the solution was poured into Petri dishes and dried in a forced air circulation oven (Rainbow®) for 48h.

Chitosan membrane sterilization. After preparation, all membranes were reallocated in pre-perforated 90x15mm Petri dishes, packed in medical grade paper, and sterilized with ethylene oxide or by ultraviolet radiation.

In vivo test. Twelve grade horses with the following characteristics were used: average weight of 400±45kg, mean age of 6±2 years, considered healthy after clinical and hematological evaluation, and fed 4kg commercial horse feed (FZEA-USP, Pirassununga/SP), 2% body weight coast-cross grass hay, and mineral salt (FZEA-USP, Pirassununga/SP) and water *ad libitum* daily.

Chitosan membranes were implanted in the right and left lateral dorsal abdominal region (flank) of the horses. Three horses received commercial chitosan (CC) membranes sterilized with ethylene oxide (CCEO, n=3); three horses received CC membranes sterilized by ultraviolet radiation (CCUR, n=3); three horses received

silver nanoparticle-impregnated CC membranes sterilized with ethylene oxide (CCSNEO, n=3); and three horses received silver nanoparticle-impregnated CC membranes sterilized by ultraviolet radiation (CCSNUR, n=3). Similarly, six horses received squid gladium chitosan (SC) membranes sterilized with ethylene oxide (SCEO, n=6) or by ultraviolet radiation (SCUR, n=6). The membranes were assessed for 15 days after the surgical procedure. The same animals were randomly used in two experimental groups, with a minimum interval of 60 days between procedures, respecting the fact of only one flank side, left or right, be under evaluation by experimental period.

During the surgical procedure, the animals were kept in the stable contained in squeeze chutes. After extensive hair trichotomy of the left or right flank regions, the animals were sedated with 10µg.kg⁻¹ detomidine (Syntec, Santana do Parnaíba/SP), intravenous (IV), followed by infiltration local anesthesia with 2% lidocaine (Cristália, Itapira/SP) in inverted-L block. Antisepsis was performed using povidone-iodine and 70% alcohol. A 10cm dorsoventral skin incision was made beginning 5cm below the transverse processes of the lumbar vertebrae and 5cm caudal to the last rib, followed by blunt subcutaneous tissue division and exposure of the external abdominal oblique muscle. The membranes were soaked in physiological solution in a stainless steel tank for 5min and then placed on the subcutaneous tissue. Subsequently, the skin was sutured with simple separate needle stitches using #1 surgical nylon thread (Sertix, Rio de Janeiro/RJ).

After the surgical procedure, antibiotic therapy with 20,000IU/kg benzathine penicillin (Zoetis, São Paulo) was conducted every 48h, intramuscular (IM), for five days, and analgesia with 1.1mg/kg flunixin meglumine (MSD, Cruzeiro/SP), IV, was performed every 24h for three days. Surgical wounds were treated with topical povidone-iodine and Hipoglós® (Johnson & Johnson, São Paulo/SP) was applied around them daily. The stitches were removed 10 days after the surgical procedure.

Physical assessment and evaluation of the implant region were performed prior to the surgical procedure (baseline) and after one (D1), two (D2), three (D3), four (D4), seven (D7), 10 (D10), and 15 (D15) days postoperatively.

The physical assessment consisted of measurement of heart rate by heart auscultation with stethoscope, respiratory rate by observation of rib cage motion, rectal temperature using a digital thermometer, intestinal motility by abdominal auscultation of the four abdominal quadrants, and mucosa staining and capillary filling time by inspection of the oral mucosa.

The implant region was evaluated for volume and temperature increase, leakage of suture exudate, and wound dehiscence. In addition, palpation around the skin suture was performed to identify tissue consistency, which was classified with fluctuation, normal, or with edema. The degree of pain observed on palpation was identified and classified as absent, mild, moderate, or severe.

Blood samples were collected from the jugular veins of the horses into tubes containing EDTA anticoagulant for blood count and fibrinogen measurement at baseline, D7 and D15.

Ultrasound assessment. An ultrasonographic evaluation (Mindray® M5VET) was performed in the implant region using a 10MHz microconvex probe immediately after the surgical procedure (baseline), and after seven (D7) and 15 (D15) days. In addition to the ultrasonographic aspects, the distance between the skin and the peritoneum was measured considering the presence of edema, clots, and exudate.

Histopathological evaluation. Fifteen days after the surgical procedure (D15), the animals were contained in squeeze chutes in the stable, and hair trichotomy and skin antisepsis were performed, followed by sedation with detomidine (10µg.kg⁻¹) (IV) and infiltration local anesthesia with 2% lidocaine in inverted-L block of the implant region. A 1cm³ fragment of subcutaneous tissue was collected, fixed in 10% buffered formalin, and stained with hematoxylin and eosin (HE) for histopathological evaluation. The skin wound was sutured with simple separate needle stitches using #1 nylon surgical thread.

Statistical analysis. Parametric data obtained from the experimental procedure were analyzed using the Statistical Analysis System software, with prior verification of normality of residues by the Shapiro-Wilk test (PROC UNIVARIATE). Variables that did not meet the statistical assumptions underwent log(X+1) transformation. Original or transformed data were submitted to analysis of variance (ANOVA) when this procedure was necessary. Repeated-measures ANOVA were conducted over time for the various sampling moments. The probabilities of interactions with time were determined by the Greenhouse-Geisser test using the REPEATED command generated by the MIXED procedure (SAS PROC MIXED). The Tukey's test was applied to find the means with significance level of 5%.

Nonparametric data were evaluated descriptively regarding tissue reactions caused by the presence of the chitosan membrane in the subcutaneous space of the flank of the horses.

RESULTS

The membranes were successfully developed and sterilized according to the previously described techniques and implanted uneventfully on the flank of the horses. No changes were observed in the physical and laboratory evaluations during the experimental period, and the values found remained within the physiology for the species.

Regarding the macroscopic evaluations of the implant areas, 100% of the animals in the CCEO (n=3), CCUR (n=3), CCSNEO (n=3) and CCSNUR (n=3) groups presented circular volume increase at D1 and D2. As of D3, the following symptoms were observed: increase in temperature, presence of edema and serosanguinolent secretion, and moderate pain on palpation, with no difference between the experimental groups. All animals in these groups presented surgical wounds with suture dehiscence and implant expulsion (Fig.1) between D7 and D10. The expelled membranes underwent microbiological evaluation, and none of them showed growth of microorganisms in culture. At D0, the ultrasonographic examination showed the presence of the membranes in the subcutaneous tissue observed by formation of a hyperechoic line and clots. At D7, anechoic images were visualized between the skin and the muscles, characterizing fluid accumulation, and the membranes could be identified. All animals under study (n=12) presented significant increase in the distance between the skin and the peritoneum, namely, D0=2.04±0.6 and D7=4.5±1.2, caused by skin edema and presence of free fluid in the subcutaneous tissue. At D15, no ultrasound evaluation was performed since the membranes had been expelled. The skin wounds healed with the application of daily topical dressings by secondary intention.

As for the animals in the SCEO (n=6) and SCUR (n=6) groups, a slight increase in volume in the implant region was observed at D1, disappearing at D2. No temperature increase, exudate, or pain on palpation in the implant region were observed in any of the animals at the experimental times. Ultrasound



Fig. 1. (A) Illustration of the commercial chitosan membrane (CC), (B) implantation of CC membrane in equine subcutaneous tissue, (C) subcutaneous swelling, (D) wound dehiscence in the CCEO group at D10, (E) CC membrane at D10, (F) squid gladius chitosan membrane (SC), (G) membrane implantation in equine subcutaneous tissue in the SCEO group, (H) surgical wound healing at D15 in the SCEO group.

assessment at D0 identified the membranes and clots in the subcutaneous tissue. At D7, it was possible to visualize the presence of the membrane in the subcutaneous tissue without fluid accumulation or tissue edema. Reduction in the distance between the skin and the peritoneum was observed ($n=12$) over the experimental period, namely, $D0=1.4\pm0.5$, $D7=1.2\pm0.5$ and $D15=0.9\pm0.2$, with no significant changes. At D15, it was not possible to identify the membranes in any animal in the experimental groups, with no changes in ultrasound image associated with skin, subcutaneous tissue and musculature. In 100% of the animals, healing of the skin sutures by first intention was observed, with removal of the stitches on D10 (Fig.1).

Histopathological examination was not performed in the animals in the CCEO, CCUR, CCSNEO and CCSNUR groups because the membranes, free in the subcutaneous tissue, were expelled through the open wounds after suture dehiscence. In the animals in the SCEO and SCUR groups, at D15, it was not possible to localize the membranes implanted in the subcutaneous tissue, and tissue fragments present between the skin and the muscles were collected. Histopathological evaluation revealed minimal to absent inflammatory reaction in the subcutaneous tissue, with small number of macrophages, and no other cell type characterizing changes in tissue was verified. No significant differences were observed between the samples.

DISCUSSION

This study demonstrated that squid gladius chitosan membranes present good acceptance in horses when implanted in subcutaneous tissue, regardless of the form of sterilization, either with ethylene oxide or by ultraviolet radiation.

In contrast, commercial chitosan membranes were not accepted in the subcutaneous tissue of the horses, resulting in rejection of the biomaterial, observed by the increase in temperature during the experimental period, formation of edema, and presence of pain on local palpation, as well as increased distance between the skin and the peritoneum

at ultrasound assessment, and presence of exudate, wound dehiscence and implant expulsion.

Impregnation of chitosan membranes with silver nanoparticles aims at providing a greater antibacterial effect, because the silver ions (Ag^+) bind to the membranes of the bacterial cells causing rupture of their walls, and are transported inside the cell, binding to DNA and RNA proteins, and interfering with energy production, enzyme function, and cell replication. In addition, by reducing the bacterial burden, silver reduces the inflammatory reaction by decreasing the activity of metalloproteinase, proinflammatory cytokines and cellular apoptosis (Leaper 2012, Shao et al. 2017). In this study, this alternative was not interesting *in vivo*, since 100% of the implanted animals rejected this biomaterial.

Although good *in vitro* results have been obtained in the manufacturing of commercial chitosan membranes impregnated or not with silver nanoparticles, when tested *in vivo* in the subcutaneous tissue of horses, they presented characteristic behavior, causing foreign body inflammatory reaction (Anderson et al. 2008) and rejection. In addition, the form of sterilization, with ethylene oxide or by ultraviolet radiation, was not responsible for the reaction to the implanted material, considering that all animals that received commercial chitosan implants showed rejection. Moreover, the expelled membranes tested negative in microbiological culture.

Therefore, a difference was observed regarding the type of chitosan with respect to the *in vivo* acceptance of the membranes when implanted in the subcutaneous tissue of horses. This finding was possibly associated with the difference in physical structure observed between them. Commercial chitosan is derived from α -chitin, found in rigid structures such as arthropod cuticles, and in these cases, a strong association with proteins and/or inorganic materials occurs. Thus, there is difficulty in standardizing the characteristics of chitin in commercial chitosan in relation to the purity and quality of chitosan, even when using the same raw material and manufacturer. Moreover, α -chitin, in addition to being the most abundant form, is considered to be more stable, since it corresponds to a dense packing of polymeric chains,

which favors the existence of numerous inter- and intra-chain hydrogen bonds. In contrast, β -chitin occurs in flexible structures, although also resistant, being the main component of squid pens. In the case of β -chitin, strands belonging to different lamellae are arranged in parallel, which hinders the establishment of hydrogen bonds involving adjacent lamellae chains and results in a less packaged material. This morphology presents relatively weak intermolecular forces, facilitating solubility in various solvents, as well as greater swelling and higher chemical reactivity (Kurita et al. 1993, Chandumpai et al. 2004, Jang et al. 2004, Campana-Filho et al. 2007). It is thus hypothesized that these differences in characteristics between α -chitin and β -chitin may have favored the non-recognition of the squid gladius implant as a foreign body by the horses of this study.

In vitro studies present numerous favorable characteristics regarding the use of chitosan *in vivo*, especially as an aid to wound healing (Berger et al. 2004, Alemdaroğlu et al. 2006, Campos et al. 2006, Azevedo et al. 2007, Brown et al. 2009, Muzzarelli 2009, Ganesh et al. 2016). In horses, there are studies that show positive results with the use of chitosan membranes on skin wounds (Martins et al. 2013). In contrast, no studies evaluating chitosan membranes when implanted in the subcutaneous tissue of horses were found in the literature. Kuhl et al. (2017) demonstrated, in rabbits, the non-integration of chitosan implants in bone failures. Shao et al. (2017) revealed an inflammatory reaction after subcutaneous implantation of chitosan membranes in rabbits, and reported the intact permanence of these membranes after 12 weeks of implantation. All of these studies were conducted with commercial chitosan.

The present study demonstrated that squid gladius chitosan membranes are biocompatible and biodegradable, and are metabolized by enzymes such as lysozyme (Muzzarelli 1997) when implanted in horses, considering that the membranes could not be localized in the subcutaneous tissue 15 days after the surgical procedure. Histopathological evaluation confirmed this finding, and showed no material suggestive of the presence of the membrane, as well as no cellularity that characterized foreign body inflammatory reaction, suggesting acceptance of the biomaterial.

Further studies addressing the use of squid gladius chitosan membranes implanted in tissues should be conducted in order to evaluate their practical applications in equine medicine, especially in assistance with incisional infections.

CONCLUSION

We conclude that squid gladius chitosan membranes are well accepted when implanted in the subcutaneous tissue of horses, being biocompatible and biodegradable, and suggest that this acceptance is due to the fact that this is a β -chitin material. Commercial chitosan membranes implanted in the subcutaneous tissue of horses result in rejection of the material.

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

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Transition period produces changes in blood and body composition in mares¹

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ABSTRACT.- Silva M.O., Manso Filho H.C., Ribeiro B.M., Ermita P.A.N., Monteiro L.C., Costa C.M., Alves S.R. & Ribeiro Filho J.D. 2019. **Transition period produces changes in blood and body composition in mares.** *Pesquisa Veterinária Brasileira* 39(10):843-848. Departamento de Veterinária, Universidade Federal de Viçosa, Av. Peter Henry Rolfs s/n, Campus Universitário, Viçosa, MG 36570-900, Brazil. E-mail: michelineozana@gmail.com

Clinical and metabolic evaluation is necessary for the monitoring of pregnant and lactating mares, as they reflect the health of the animal. The body condition of the mare is an indicator of reproductive efficiency. The study aimed to determine the possible variations in body and metabolic condition in Mangalarga Marchador mares during the transition period. Forty-eight mares distributed in two groups were used: Maintenance Group (MG), composed of non-pregnant and non-lactating mares, and Transition Group (TG), formed by pregnant mares and who after delivery became lactating. Analyses were performed in the times T-60, T-30 and T-15 before delivery, first six hours (T0) after delivery and T15, T30 and T60 days after delivery. MG was evaluated only at one time (T-60). Body weight and fat-free mass differed ($P<0.05$) between the groups. The percentage of fat was lower in MG. Mares had a higher fat percentage in TG at T-60 and T-30 times. There was a difference ($P<0.05$) in the amount of cholesterol between MG and TG (T0, T15 and T30). Triglycerides were different between the groups. TG showed higher concentrations of non-esterified fatty acids ($P<0.05$). There was a higher amount of glucose in TG (delivery and lactation) when compared to mares in MG. Changes in body condition and metabolic constituents occurred in the animals resulting from physiological adaptations of the transition period. The energetic components are the most affected from the transition period, with intense fat mobilization to supply the body demands.

INDEX TERMS: Transition period, blood, body composition, mares, clinical evaluation, Mangalarga Marchador, pregnancy, percentage of fat, equine.

RESUMO.- [Período de transição produz alterações na composição do sangue e do corpo em éguas.] Avaliações clínicas e metabólicas são necessárias para o monitoramento de éguas gestantes e lactantes, pois refletem a saúde do animal. A condição corporal da égua pode ser um indicador da eficiência reprodutiva. O estudo objetivou determinar as

possíveis variações na condição corporal e metabólica em éguas Mangalarga Marchador durante o período de transição. Foram utilizadas 48 éguas distribuídas em dois grupos: Grupo em Manutenção (GM) composto por éguas não gestantes e não lactantes; Grupo em Transição (GT) formado por éguas gestantes e que após o parto tornaram-se lactantes. As análises foram realizadas nos tempos T-60, T-30 e T-15 antes do parto, primeiras seis horas (T0) após o parto e T15, T30 e T60 dias após o parto. O GM foi avaliado apenas uma vez (T-60). O peso corporal e a massa livre de gordura diferiram ($P<0,05$) entre os grupos. O percentual de gordura foi menor em GM. As éguas apresentaram maior porcentagem de gordura no GT no T-60 e no T-30. Houve diferença ($P<0,05$) na quantidade de colesterol entre GM e GT (T0, T15 e T30). Triglicérides foram diferentes entre os grupos. GT apresentou maiores

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concentrações de ácidos graxos não esterificados ($P < 0,05$). Houve maior quantidade de glicose no GT (parto e lactação) quando comparada às éguas no GM. Mudanças na condição corporal e nos constituintes metabólicos ocorreram nos animais resultantes de adaptações fisiológicas do período de transição. Os componentes energéticos são os mais afetados neste período, existindo intensa mobilização de gordura para suprir demandas corporais.

TERMOS DE INDEXAÇÃO: Período de transição, composição do sangue, corpo, éguas, avaliação clínica, Mangalarga Marchador, gravidez, porcentagem de gordura, sangue, equinos.

INTRODUCTION

The time interval between the end of pregnancy, delivery, and early lactation is called the transition period (Manso Filho et al. 2008). In mares, successive changes occur in the maternal organism during pregnancy, typically in the anabolic phase, in preparation for the end of pregnancy, parturition and early lactation, which represent the catabolic phase. The metabolic events are known to be adaptive mechanisms of the body in the transition from pregnancy to lactation (Hura et al. 2017). These continuous changes in the anabolism-catabolism cycle are dependent on the management system and affect the metabolism of all nutrients to ensure that the nutritional requirements of the fetuses and newborns are being met. These important events are relevant for the formation of a future athlete horse.

Understanding the relationship between body fat and reproductive efficiency of embryo donor mares during strategic phases of equine physiology allows the creation of a nutritional and management program appropriate to their body conditions. Deposition of adipose tissue differs between pregnant and nonpregnant mares and causes changes in anatomical conformation. In pregnant mares, the layer of fat is reduced in the dorsal region, ribs, and the base of tail, being most prominent in the region of the withers of scapula (Henneke et al. 1983).

Assessment of body condition is used to determine the reproductive efficiency in mares (Rodrigues et al. 2011) and when combined with laboratory tests, improves reproductive efficiency among mares and survival rates of newborns. To assess the body condition of breeding mares, different methods are available and can be used, such as the determination of the body score index, which is a subjective method of assessing body fat and muscles (Henneke et al. 1983). Furthermore, the determination of the body fat percentage has recently been carried out using ultrasound equipment, providing an objective measure that is efficiently replicable by veterinarians. Knowledge of the body fat percentage can contribute to the assessment of nutritional management and training practices (Manso Filho et al. 2009).

Body modifications are associated with changes in different blood biomarkers, the main ones being related to the energy metabolism of both carbohydrates and fats. An example is the loss of lean body mass during lactation, which may indicate the occurrence of protein mobilization of the skeletal muscle to provide amino acid precursors for milk and gluconeogenesis required for the synthesis of lactose, however, this loss might be harmful to the mare's future performance (Manso Filho et al. 2008).

In view of the fact of the importance of the metabolic status relationship between mare and their fetuses/newborns and because of the scarcity of available studies addressing the topic, the present study aimed to determine the variations in body and metabolic condition in Mangalarga Marchador mares during the transition period.

MATERIALS AND METHODS

Ethics statement. The study was conducted in Salgada Farm, located in the city of Itaperuna, Rio de Janeiro, Brazil. The study was approved by the Committee on Ethics in the Use of Animals (CEUA) of the "Universidade Federal de Viçosa" (protocol no. 95/2015).

Animals and breeding system. Forty-eight Mangalarga Marchador mares (aged 5 to 15 years) were distributed in two groups: Maintenance Group (MG), composed of non-pregnant and non-lactating mares, and Transition Group (TG), formed by pregnant mares and who after delivery became lactating. At the farm, the animals were kept under extensive management. All mares that were selected in the study were kept in pastures, with Tifton grass (*Cynodon* spp.), water and commercial mineral salt (Coequi Plus, Tortuga) available ad libitum, without supplementation of concentrates. Two weeks prior to the delivery, the mares were transferred to maternity paddocks, maintaining the diet, where they were kept until the time of delivery.

Evaluation of body condition. All mares were weighed on a calibrated scale with a measuring capacity of up to 1.000kg. The body fat percentage was determined by the measurement of the thickness of the fat layer in the rump, as described by Manso Filho et al. (2009). An ultrasound (SonoSite 180 PLUS, Sonosite FujiFilm, Sao Paulo/SP, Brazil) with a 10-5MHz linear transducer was used. A midpoint was drawn between the tuberosity of the ischium and the ilium, approximately 10cm from the midline of the body, and at this point, the fat layer (FL) was measured. The measurement was always on the same side and was made by the same evaluator. Body fat percentage (BFP) was obtained as follows: $[BFP = 8.64 + (4.7 \times FL)]$. Thus, the fat-free mass was determined by calculating the body weight difference by the body fat percentage of each mare, and the results were presented in kilograms (kg).

Metabolic profile. Blood samples for serum and plasma obtain were collected at seven times: T-60, T-30, T-15, T0 (first 6 hours after delivery), T15, T30 and T60. The samples were taken without prior fasting using disposable needles. Flasks containing sodium fluoride were used to obtain plasma and siliconized vials without anticoagulant were used for serum acquirement. The samples were processed, aliquoted into microtubes and immediately frozen at -20°C until the time of the laboratory analysis. Biochemical analyses were performed on a Humastar 300 automatic device (Humano, In Vitro Diagnóstica Ltda., Itabira/MG, Brazil).

The serum total cholesterol (Enzymatic Colorimetric Method with Lipid Clearing Factor, In Vitro Diagnóstica Ltda, Itabira/MG, Brazil), triglycerides (Colorimetric Enzymatic Method with Lipid Clearing Factor, In Vitro Diagnóstica Ltda, Itabira/MG, Brazil), and non-esterified fatty acid (NEFA) (Colorimetric Method, Randox, Centerkit, Ribeirão Preto/SP, Brazil) levels and the plasma concentrations of glucose (Enzymatic Colorimetric Method without deproteinization, In Vitro diagnóstica Ltda, Itabira/MG, Brazil) were measured.

The body condition of TG mares was evaluated at the following time points: T-60 (60 days before delivery), T-30 (30 days

before delivery), T-15 (15 days before delivery), T15 (15 days after delivery), T30 (30 days after delivery) and T60 (days after delivery). MG mares were evaluated only once (first collection of all "T-60" animals).

Statistical analysis. To analyze the data, the Statistical Analysis System (SAS) was used (SAS 2002). The data were evaluated using the Kolmogorov-Smirnov and Bartlett's tests to verify the normality of the errors and homogeneity of variances, respectively, and the variables that did not satisfy the ANOVA assumptions were submitted to the square root transformation [$y' = \sqrt{y + 0.5}$].

The data for pregnant mares were analyzed in linear mixed models with repeated plots in time using PROC MIXED (SAS 2002), with autoregressive and animal covariance structure as random effect (Littell et al. 2006), according to the mathematical model: $Y_{ij} = X_{ijk}\beta_k + Z_{ijk}b_{ik} + e_{ij}$. For the comparison of means (least square means), the t-test with Tukey-Kramer adjustment was used for the low instability variables (CV<15%), and the means were adjusted by the t-test for unstable variables (CV>15%). In addition, the means of the times were compared to the MG using the Dunnett's test. The level of significance was $\alpha = 0.05$.

RESULTS

During the entire experimental period, mean ambient temperatures (minimum and maximum) were 17.4°C and 28.6°C, respectively, and the mean relative humidity of the air was 49%. The evaluations were always performed from 9 to 11 a.m. and by the same evaluators. No interurrences with the mares (abortion, inflammation, infections or parasitic diseases) were reported that could compromise the study.

The body weight (Fig.1) and fat-free mass (Fig.2) of the mares showed decreased values during the study. A difference was observed in both MG variables (P<0.05) when compared to TG at T-60, T-30, and T-15 times (pregnant mares). Over time, body weight and fat-free mass decreased progressively (P<0.05). The percentage of body fat (Fig.3) was lower in MG mares. A difference between MG (P<0.05) and TG at T-60 and T-30 times was observed. In the evaluation over time (P<0.05), the animals of the T-60 and T-30 showed higher percentage of fat than the animals of times T-15, T15, T30, and T60, that maintained the percentage of fat without change.

The serum cholesterol concentration was different (P<0.05) between MG and TG at T0, T15, and T30 times, with a higher mean value observed in the first six hours postpartum (T0). The mares of the MG and TG groups (T-60, T-30, T-15, T0, and T60) showed average concentration within the reference values for the equine species. Mares evaluated at T15 and T30 times showed a cholesterol concentration below the reference values. A difference (P<0.05) was found over time in the animals evaluated in the transition period (Table 1).

The concentration of triglycerides remained within the reference values for the equine species. A difference (P<0.05) was observed between the MG and TG mares (T-30, T-15, T0, T15, and T30) (Table 1). In the evaluation over time, the mares of TG showed a progressive increase (P<0.05) in the amount of triglycerides in the T-60, T-30, and T-15 periods, showing a higher value at T-15 when compared with the other time periods evaluated. In the T0 (P<0.05) a

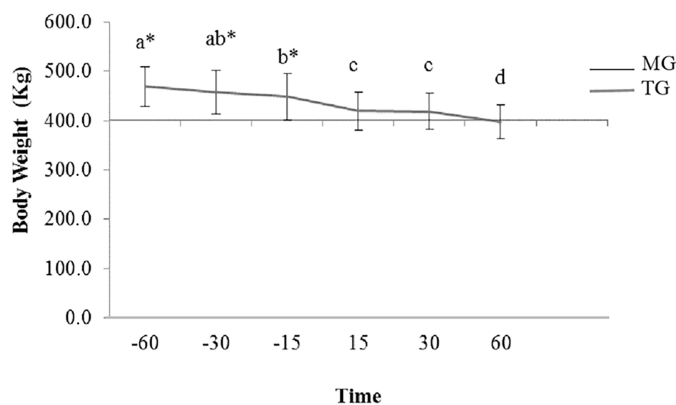


Fig.1. Mares body weight in maintenance (MG) and transition (TG) group. Lowercase letters differ the TG over time (P<0.05) by the Tukey test. Letters followed by * differ (P<0.05) from the MG by the Dunnett test.

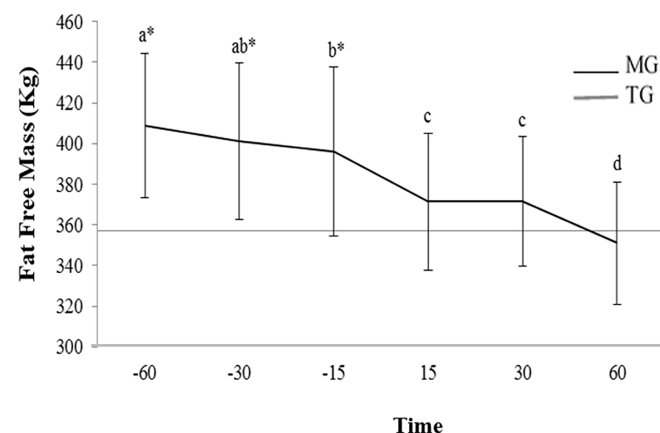


Fig.2. Mares fat free mass in maintenance (MG) and transition (TG) group. Lowercase letters differ the TG over time (P<0.05) by the Tukey test. Letters followed by * differ (P<0.05) from the MG by the Tukey test.

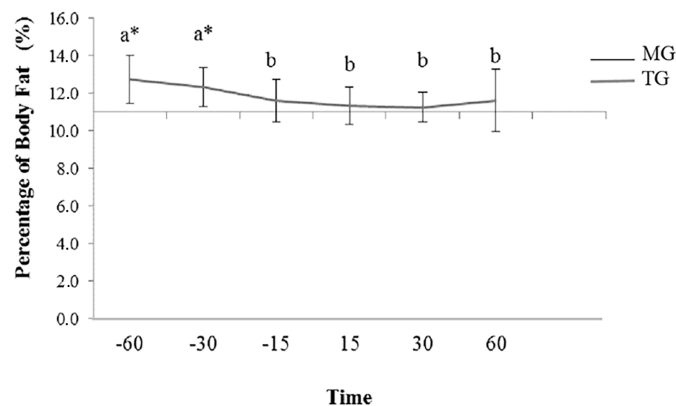


Fig.3. Percentage of body fat of mares in maintenance (MG) and transition (TG) group. Lowercase letters differ the GT over time (P<0.05) by the Tukey test. Letters followed by * differ (P<0.05) from the MG by the Dunnett test.

Table 1. Mean values and standard deviation of the concentrations of cholesterol - CHOL (mmol/L), triglycerides - TRIGLY (mmol/L), non-esterificate fatty acids - NEFA (mmol/L) and glucose - GLU (mmol/L) evaluated in the Transition Group (TG) and Maintenance Group (MG)

Blood parameters	Times - TG							MG
	Pregnancy			Delivery	Lactation			
	T-60	T-30	T-15	T0	T15	T30	T60	
CHOL	2.3 ± 0.3 ^b	2.5 ± 0.5 ^{ab}	2.5 ± 0.5 ^b	2.9 ± 0.6 ^{Aa}	1.7 ± 0.3 ^{Ac} ^d	1.6 ± 0.3 ^{Ad}	1.9 ± 0.3 ^c	2.3 ± 0.3
TRIGLY	0.3 ± 0.1 ^b	0.5 ± 0.2 ^{Aab}	0.5 ± 0.2 ^{Aa}	0.4 ± 0.2 ^{Aab}	0.1 ± 0.0 ^{Ac}	0.1 ± 0.0 ^{Ac}	0.1 ± 0.0 ^c	0.2 ± 0.2
NEFA	0.3 ± 0.1 ^{bc}	0.5 ± 0.2 ^{Aab}	0.3 ± 0.2 ^{abc}	0.5 ± 0.2 ^{Aa}	0.4 ± 0.2 ^{Aabc}	0.4 ± 0.2 ^{Aabc}	0.2 ± 0.1 ^c	0.2 ± 0.1
GLU	5.3 ± 0.4 ^{ab}	5.5 ± 0.4 ^{ab}	5.0 ± 0.4 ^b	5.6 ± 1.1 ^{Aa}	4.1 ± 0.4 ^{Ac}	3.9 ± 0.4 ^{Ac}	4.1 ± 0.5 ^{Ac}	5.1 ± 0.4

Means followed by lower case letters on the same line differ ($P < 0.05$) by the Tukey test, means followed by capital letters in the same row differ ($P < 0.05$) in the MG by Dunnet's test.

reduction in triglycerides was noted, the lowest values being observed at T15, T30, and T60.

The lowest serum concentration of NEFA ($P < 0.05$) was obtained in MG when compared to TG (Table 1). A difference ($P < 0.05$) was found in the TG animals at all evaluated times, with a higher amount in the T0. During the lactation phase (after T0), there was a progressive reduction of the values.

A difference ($P < 0.05$) in the plasma glucose concentration between the MG and TG groups at T0, T15, T30, and T60 was found. Higher amount of glucose was observed at T0. Over time, a difference ($P < 0.05$) was found between the times evaluated (Table 1). The mares evaluated at T15, T30, and T60 showed values below the reference values for the equine species.

DISCUSSION

In the transition period, significant changes occurred in the body condition and in the metabolic components of the mares. The results showed that the final third trimester of pregnancy, delivery, and onset of lactation promote changes in the maternal organism, compatible with the results found in other studies (Harvey et al. 2005, Bazzano et al. 2014, Mariella et al. 2014). Knowledge of these changes is fundamental for the identification of diseases that can compromise the health of the mare, fetus and development of the foal.

The good health condition of the mares during the experimental period allowed a more accurate evaluation of the body and metabolic condition of the animals. This is the first study that evaluated Mangalarga Marchador mares during the transition period, although several studies have been carried out with the same purpose in mares of different breeds and during different stages of pregnancy, delivery and lactation (Hunka et al. 2015, Marchiori et al. 2015).

To produce valuable animals, some breeders changed the animal management, such as feeding with highly energetic food (Paz et al. 2013), which may predispose the breeding animal to develop metabolic disorders and obesity (Marchiori et al. 2015). Therefore, body weight, fat percentage and fat-free mass evaluation during the transition period in mares is crucial to make appropriate changes in the management of the animals to avoid the development of disorders.

Weight gain during pregnancy is caused by fetal growth, placental development and increased deposition of adipose tissue to achieve energy demands at the end of gestation and during lactation. However, in the transitional period, the progressive loss of body weight and the percentage of body

fat around parturition is due to the negative energy balance during in this period. Reduced food intake, with increased fetal metabolic demands, foaling, and milk production to provide nutritional requirements for foals (Hura et al. 2017) may be one of the major reasons for weight and body fat losses. Animals submitted to higher metabolic demands have lower fat percentages (Manso Filho et al. 2009). The first study identifying changes in body composition in mares during the transition period was conducted by Manso Filho et al. (2008). The authors realized that during lactation, mares lost lean body mass as they maintained their body fat and that this fact can be explained since late gestation represents a mild catabolic state, that intensifies after delivery due to lactation. Similar findings were observed in the present study.

Marchiori et al. (2015) revealed a linear increase in the body weight of Crioulo mares from eight to eleven months. The authors suggested that weight gain that occurs in pregnant mares in the final third trimester of pregnancy, leads to a reserve of energy that is meant for use during lactation. This did not occur in our study, wherein a progressive reduction in the mean body weight values from T-60 to T60 was observed. Lawrence et al. (1992) suggest that pregnant mares may increase their body fat reserve during mid-gestation and then mobilize part of this reserve until delivery. These studies did not evaluate the food intake of the animals, as in the present study. For this reason, it cannot be affirmed that weight gain is a consequence of increased food intake. However, it is clear that in the last trimester of pregnancy and onset of lactation, a progressive loss of body weight is observed, which may be due to the mobilization of energy reserves owing to higher fetal demand.

Pregnancy and lactation resulted in increased metabolic requirements. Although homeostatic mechanisms act to maintain the concentration of nutrients in the blood at a relatively constant level, some variations are likely to occur (Harvey et al. 2005). According to Hunka et al. (2015), the lactating period is a challenge for mares, as the number of nutrients available in breast milk must meet the nutritional demands of foals.

Cholesterol is an important sterol in mammals, playing an important role in several physiological stages (Vasilenko 2016). The concentration of cholesterol in the blood can suffer influence from factors such as the amount of food that is ingested and absorbed as well as the portion synthesized in the liver and the amount that is reabsorbed (Aoki & Ishii 2012). The cholesterol decreases slightly early in gestation

and then increases progressively. Its concentration at the end of pregnancy rises by approximately 50% when compared to that in the non-pregnant state. However, these values slowly return to normal (15 to 20%) by the second week postpartum (Hadden & McLaughlin 2009).

Initially, the reduction in the amount of cholesterol during gestation may be the result of factors such as plasma hemodilution, decreased secretion of liver lipoproteins, transport of cholesterol from the maternal body to the fetus and the increased use for the biosynthesis of steroid hormones. According to Vasilenko (2016), it is probable that the increase in cholesterol concentration is necessary for an increased rate of synthesis of hormones during pregnancy; besides, the high cholesterol observed during pregnancy may also be secondary to the increase in hormones associated with pregnancy. The peak of serum cholesterol concentration was in the first six hours after delivery and its progressive reduction during the first 30 days after lactation is reflective of the increased energy demand in mares.

Triglyceride is a key nutrient at the transition period, as it is the main component of adipose tissue and milk (Aoki & Ishii 2012). In the last trimester of pregnancy, higher concentrations of this lipid were observed owing to the greater anabolic activity and increase in fat metabolism, necessary for fetal growth (Vincze et al. 2015). In a study by Harvey et al. (2005), significant differences were observed in different variables of mares when comparing pregnancy with the lactation period. The total concentration of triglycerides was reduced during lactation. Bazzano et al. (2014) also observed a significant reduction in the triglyceride levels after delivery. Although these studies differed with regard to breeds, times, and management, the concentration of triglycerides was similar to that obtained in this study. Hadden & McLaughlin (2009) explain that triglyceride levels decrease gradually after delivery, returning to non-gestational values close to the second week postpartum. The concentrations of NEFA are used as indicators of lipid degradation in adipose tissue (Aoki et al. 2016) and reflect the status of the energy balance of the body (Hura et al. 2017). When animals enter a negative energetic balance, the organism signals the need to mobilize stored energy, the secretion of epinephrine occurs, which acts on adipose tissue, potentiating fat breakage and a resultant increase in NEFA values (Frape 2004, Aoki & Ishii 2012). In addition, within about three days after calving, the free fatty acid levels fall to non-pregnant values (Hadden & McLaughlin 2009). On the basis of other studies, Hura et al. (2017) explain that the increase in the concentration of NEFA and reduction in triglyceride levels after birth prove that mares compensate the negative energy balance by mobilization of fat reserves.

The glucose concentration in the fetus is 15% to 20% lower than in the mother (Hadden & McLaughlin 2009) and in this way, facilitates the glucose transport from mother to fetus to supply the energetic demands. Glucose is the main component for fetal growth; however, during the end of gestation, its distribution remains constant, because the fetus begins to require energy from other substrates such as lactate and amino acids (Manso Filho et al. 2008).

Mares use glucose as a source of energy, and a change in their concentration may be due to an imbalance in the regulatory hormone (e.g., insulin) or excessive intake of

energy sources. Mariella et al. (2014) explain that a higher concentration of glucose occurs at birth. The increase in the glucose concentration in mares may be associated with the physical stress which the animals are subjected during foaling (Aoki & Ishii 2012). According to Vincze et al. (2015), changes in glucose regulation during pregnancy may be associated with the development of insulin resistance as well as a secondary increase in the plasma triglyceride concentration. The decrease in the concentration of glucose from lactation, in turn, is because of the greater demand by the mammary gland for obtaining energy and for lactose synthesis (Mundim et al. 2007).

CONCLUSIONS

It is possible to conclude that the changes in the body condition and metabolic components of the animals result from physiological adaptations during the transition period. The energetic components are the most affected from the transition period with intense fat mobilization to supply the body demands.

These evaluations are necessary to the species, during the period, as they reflect the health of the animals, allowing possible interventions in non-physiological cases.

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

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Os Artigos Originais devem conter resultados de pesquisa ainda não publicados ou submetidos para outros periódicos.

Artigos de Revisão de Literatura, submetidos a convite, devem constituir-se de análise crítica, de assuntos na área de experiência dos autores, isto é, quando os autores já tiverem publicado anteriormente artigos sobre o assunto.

Os raros Tópicos de Interesse Geral devem constituir-se de assuntos de grande importância atual baseado na vasta experiência dos autores.

As opiniões e conceitos emitidos nos artigos submetidos são de responsabilidade dos autores. O Conselho Editorial da PVB, com a assistência da Assessoria Científica, pode sugerir ou solicitar modificações. Os artigos submetidos são avaliados pelos pares (*peer review*) e, aceitos para publicação com dois pareceres favoráveis, ou rejeitados por dois pareceres desfavoráveis.

Os direitos autorais dos artigos aceitos para publicação permanecem com os autores.

1. Os artigos devem ser organizados em TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, MATERIAL E MÉTODOS, RESULTADOS, DISCUSSÃO, CONCLUSÕES (de preferência os últimos três separadamente), Agradecimentos, Declaração de conflito de interesse e REFERÊNCIAS:

a) O **TÍTULO** deve ser conciso e indicar o conteúdo do artigo; pormenores de identificação científica devem ser colocados em MATERIAL E MÉTODOS.

b) O(s) Autor(es) com numerosos primeiros nomes e sobrenomes, deve(m) padronizar o seu “nome para publicações científicas”, como por exemplo: Cláudio Severo Lombardo de Barros, escreve Cláudio S.L. Barros ou Barros C.S.L.; Franklin Riet-Correa Amaral escreve Franklin Riet-Correa ou Riet-Correa F. **Os artigos devem ter no máximo 8 (oito) autores.** O autor para correspondência deve ser um autor que garanta o contato com o Conselho Editorial da PVB. Asteriscos de chamadas para o rodapé não devem ser sobrescritos.

c) O **Cabeçalho do ABSTRACT** deve conter além dos nomes dos autores abreviados invertido, o ano, o TÍTULO, o endereço postal do laboratório (inclusive o CEP) ou instituição principal onde foi desenvolvida a pesquisa. Endereços postais brasileiros

não devem ser traduzidos para o inglês, mesmo em artigos escritos na língua inglesa, a fim de evitar dificuldade na postagem. Deve-se conferir os nomes dos autores do artigo e do Cabeçalho do Abstract para evitar discrepâncias.

d) O **Rodapé da primeira página** deve conter os endereços profissionais postais completos dos autores (evitando-se traços horizontais), na língua do país do respectivo autor (em português, espanhol, inglês) e seus e-mails; o e-mail do autor para correspondência deve ser sublinhado. Os sinais de chamada para os nomes dos autores devem ser números arábicos, colocados em sobrescrito, sem o uso automático de “Inserir nota de fim”, do Word (essas chamadas devem ser contínuas por todo artigo, isto é, em todas as notas de rodapé das outras páginas).

e) O **ABSTRACT** deve ser uma versão do RESUMO, mas pode ser mais explicativo, seguido de “INDEX TERMS” que devem incluir termos do título, por não se tratar somente de “ADDITIONAL INDEX TERMS”.

f) O **RESUMO** deve conter o que foi feito e estudado, indicando a metodologia e dando os mais importantes resultados e conclusões, seguido dos “TERMOS DE INDEXAÇÃO” que incluem termos do título, por não se tratar somente de “TERMOS DE INDEXAÇÃO ADICIONAIS”.

g) A **INTRODUÇÃO** deve ser breve, com citação bibliográfica específica sem que a mesma assuma importância principal e deve finalizar com a indicação do objetivo do artigo.

h) **MATERIAL E MÉTODOS** deve reunir a totalidade dos dados que permitam o desenvolvimento de trabalho semelhante por outros pesquisadores.

i) Em **RESULTADOS** devem ser apresentados concisamente os dados obtidos.

j) Na **DISCUSSÃO** devem ser confrontados os resultados diante da literatura. Não convém mencionar artigos em desenvolvimento ou planos futuros, de modo a evitar uma obrigação do autor e da revista de publicá-los.

k) **CONCLUSÕES** devem basear-se somente nos resultados obtidos e devem ser apresentados em diferentes parágrafos (uma Conclusão somente deve ser apresentada em parágrafo único).

l) Os **Agradecimentos** não devem aparecer no texto ou em notas de rodapé; devem ser sucintos e colocados antes da Declaração de conflito de interesse e da Lista de Referências.

m) A **Declaração de conflito de interesse** é obrigatória e deve ser mencionada nos casos positivos ou negativos; deve ser sucinta e colocada imediatamente antes da Lista de Referências.

n) A Lista de **REFERÊNCIAS** deve incluir todas as citações apresentadas no texto e que tenham servido como fonte para consulta. A Lista deve ser ordenada alfabética e cronologicamente, pelo sobrenome do primeiro autor, seguido de todos os demais autores (em caixa alta e baixa), do ano, do título da publicação citada, e abreviado (por extenso em casos de dúvida) o nome do periódico. Sugerimos consultar exemplos dos últimos fascículos (www.pvb.com.br).

(**Notem:** (1) As Referências citadas no texto devem ser colocadas em ordem cronológica, mas alfabética tratando-se de referências do mesmo ano; (2) Quando utilizados programas de formatação (p.ex. Endnote X7), remover o fundo automático cinzento antes da submissão, para não dificultar eventuais correções.

2. Na elaboração do texto devem ser atendidas as seguintes normas:

a) Fonte **Cambria, corpo 10, entrelinha simples; página formato A4, com 2cm de margens** (superior, inferior, esquerda e direita), texto corrido em uma coluna justificada, com as Legendas das Figuras no final (logo após a Lista de REFERÊNCIAS) sem repetir as legendas junto com as Figuras.

b) **ABSTRACT** e **RESUMO** serão escritos em um só parágrafo corrente e não devem conter citações bibliográficas.

c) A redação dos artigos deve ser concisa, com a linguagem, tanto quanto possível, no passado e impessoal.

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Apresentação das Figuras:

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

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

Exemplos de Referências

➤ Artigos publicados em periódicos:

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

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

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

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

➤ Livros:

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

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

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

➤ Capítulos de livros:

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

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

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

➤ Dissertações e Teses:

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

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

➤ Resumos publicados em eventos:

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

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

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

GUIDE FOR AUTHORS

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

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

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

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

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

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

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

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

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

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

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

The author rights of the accepted papers are preserved.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3. Figure presentation:

a) Save images at 300 dpi, TIF files.

b) Send each figure separately.

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

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

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

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

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

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

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

Exemples for References:

➤ Articles published in scientific journals:

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

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

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

➤ Books:

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

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

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

➤ Chapters of books:

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

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

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

➤ Dissertations and Theses:

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

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

➤ Abstracts published in Events:

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

Em 15 de agosto de 2019

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

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

(Note: Consult entire papers instead of only Abstracts)

LIVESTOCK DISEASES

- Techniques for prevention and control of poisoning by sodium monofluoroacetate (MFA)-containing plants in ruminants** [Técnicas para prevenção e controle da intoxicação em ruminantes por plantas que contém monofluoroacetato de sódio]. Pessoa D.A.N., Medeiros R.M.T. & Riet-Correa F. 771-779
- Biochemical, endocrine, and histopathological profile of liver and kidneys of sheep with pregnancy toxemia** [Perfil bioquímico, endócrino e histopatológico do fígado e rins de ovelhas com toxemia na prenhez]. Souto R.C.J., Afonso J.A.B., Mendonça C.L., Dantas A.F.M., Cajueiro J.F.P., Gonçalves D.N.A., Oliveira Filho E.F. & Soares P.C. 780-788
- Comparison of electrolyte and acid-base balances of Dorper breed ewes between single and twin pregnancies** [Comparação dos equilíbrios eletrolítico e ácido básico de ovelhas da raça Dorper entre gestação única e gemelar]. Santarosa B.P., Dantas G.N., Ferreira D.O.L., Carvalho M.G., Rodrigues M., Pereira P.F.V., Silva A.A. & Gonçalves R.C. 789-795
- Bacteria isolated from the lower respiratory tract of sheep and their relationship to clinical signs of sheep respiratory disease** [Bactérias detectadas no trato respiratório inferior de ovinos e a relação com as manifestações clínicas da doença respiratória ovina]. Franco M.F., Gaeta N.C., Alemán M.A.R., Melville P.A., Timenetsky J., Balara M.F.A. & Gregory L. 796-801
- Herbaspirillum seropedicae* as a degrading bacterium of monofluoroacetate: effects of its inoculation in goats by ingesting *Amorimia septentrionalis* and the concentrations of this compound in plants sprayed with the bacterium** [*Herbaspirillum seropedicae* como bactéria degradadora de monofluoroacetato de sódio: efeitos de sua inoculação em caprinos ingerindo *Amorimia septentrionalis* e nas concentrações deste composto em plantas pulverizadas com a bactéria]. Pessoa D.A.N., Lopes J.R.G., Souza E.M., Campos E.M., Medeiros R.M.T., Cook D., Lee S.T. & Riet-Correa F. 802-806
- Pseudomonas* spp. and other psychrotrophic microorganisms in inspected and non-inspected Brazilian Minas Frescal cheese: proteolytic, lipolytic and AprX production potential** [*Pseudomonas* spp. e outros micro-organismos psicrotróficos em queijos Minas Frescal inspecionados e não inspecionados: potencial proteolítico, lipolítico e produção de AprX.]. Teider Junior P.I., Ribeiro Júnior J.C., Ossugui E.H., Tamanini R., Ribeiro J., Santos G.A., Alfieri A.A. & Beloti V. 807-815
- Salmonella* spp. prevalence, antimicrobial resistance and risk factor determination in Colombian swine farms** [*Salmonella* spp. prevalência, resistência antimicrobiana e determinação de fatores de risco em granjas suínas colombianas]. Giraldo-Cardona J.P., Guadrón-Ramírez D., Chamorro-Tobar I., Pulido-Villamarín A., Santamaría-Durán N., Castañeda-Salazar R., Zambrano-Moreno C. & Carrascal-Camacho A.K. 816-822

SMALL ANIMALS DISEASES

- Medical therapy using omeprazole in 12 hydrocephalic dogs: clinical, diagnostic, and therapeutic findings** [Terapia médica utilizando omeprazol em 12 cães hidrocefálicos: achados clínicos, diagnósticos e terapêuticos]. Pelegrini L.F., Silva N.F., Campos O.P.S., Nery C.C., Silva F.M., Lemos R.S., Yamauchi K.C.I. & Amude A.M. 823-829
- Ganciclovir attenuates the respiratory disease induced by *Equid alphaherpesvirus 1* in rabbits** [O ganciclovir atenua a doença respiratória produzida pelo alfavírus equino 1 em coelhos]. Mortari A.P.G., Amaral B.P., De Oliveira P.S.B., Dotto E.K., Flores M.M., Cargnelutti J.F., Weiblen R. & Flores E.F. 830-836

MORPHOPHYSIOLOGY

- Behaviour of different types of chitosan membranes implanted in horses** [Comportamento de diferentes tipos de membranas de quitosana implantadas em equinos]. Rollim V.M., Reginato G.M., Fernandes L.M., Arantes J.A., Rigo E.C.S., Vercik L.C.O., Freitas S.H. & Dória R.G.S. 837-842
- Transition period produces changes in blood and body composition in mares** [Período de transição produz alterações na composição do sangue e do corpo em éguas]. Silva M.O., Manso Filho H.C., Ribeiro B.M., Ermita P.A.N., Monteiro L.C., Costa C.M., Alves S.R. & Ribeiro Filho J.D. 843-848