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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA ANIMAL

CAMPUS DE PATOS

OCORRÊNCIA DE TUBERCULOSE BOVINA NO ESTADO DO RIO GRANDE DO NORTE, BRASIL

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PATOS-PB

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PIRAJÁ SARAIVA BEZERRA NETO

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Orientador: Dr. Severino Silvano dos Santos Higino
Coorientadora: Dra. Rosália Severo de Medeiros

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“O homem é do tamanho do seu sonho”.

Fernando Pessoa

A **Deus** e ao meus pais, **Reginaldo** e **Luzia**, que são meu alicerce...

Dedico

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Sumário

Resumo

Esta dissertação é comporta por dois capítulos. No capítulo I objetivou-se determinar a ocorrência de tuberculose por *Mycobacterium bovis* em bovinos abatidos em dois abatedouros públicos da mesorregião Central Potiguar, estado do Rio Grande do Norte, Brasil. No período compreendido entre novembro de 2017 a agosto de 2018, 11,616 carcaças passaram por inspeção de rotina, onde foram observadas lesões sugestivas de tuberculose em um animal (0.009%). Amostras de tecidos localizadas no pulmão, coração e baço foram submetidas a cultivo bacteriológico e diagnóstico molecular. O omento foi utilizado para exame histopatológico e coloração de Ziehl-Neelsen. As lesões foram cultivadas por 90 dias em meio *Stonebrink,* porém não houve crescimento. Quando usada a nested-PCR, as amostras de pulmão, coração e baço foram positivas. O exame histopatológico do omento mostrou a presença de múltiplos granulomas de centro necrótico e áreas de mineralização e quando submetido à coloração de Ziehl-Neelsen, detectou-se a presença de bacilos álcool-ácido resistentes. O cultivo microbiológico, apesar de ser considerado padrão ouro, pode apresentar resultados falsos negativos, além de ser uma técnica demorada, dificultando sua utilização para vigilância em abatedouros. A presença de animal positivo no estudo representa um sério problema no que diz respeito à saúde pública, principalmente para criadores, magarefes, técnicos e para o consumidor final. A nested-PCR e a histopatologia apresentam resultados rápidos e eficazes para o diagnóstico da enfermidade. Portanto, um dos sistemas essenciais aplicados ao controle da tuberculose bovina é a vigilância epidemiológica dos animais nos abatedouros. O objetivo do capítulo II foi determinar a frequência de animais positivos para a tuberculose bovina no estado do Rio Grande do Norte. Os dados foram fornecidos pelo Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte (IDIARN) e são oriundos de suas Unidades Locais de Sanidade Animal e Vegetal (ULSAV'S) coletados dos relatórios mensais emitidos por médicos veterinários habilitados para atuação no âmbito do Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (PNCEBT), compreendendo o período de junho de 2012 a junho de 2018. Para o diagnóstico foi utilizada, como prova de triagem, o teste cervical simples e como prova confirmatória o teste cervical comparativo. No total, foram testados 16.889 bovinos, dos quais 44 animais (0,26%) apresentaram resultado positivo. Não foi observada diferença significativa ($P < 0,05$) na frequência de positividade entre fêmeas (0,25%) e machos (1,16%). Frente a este resultado e considerando a importância da bovinocultura local para a economia, é importante a condução de medidas que incluam a conscientização dos produtores, controle sanitário na aquisição e venda de matrizes, fiscalização nas barreiras sanitárias e levantamentos periódicos da situação epidemiológica desta doença, principalmente nas unidades com maior frequência de animais positivos, com o objetivo de evitar, ou pelo menos minimizar, a disseminação do agente.

Palavras-Chave: Tuberculose, *Mycobacterium bovis*, Rio Grande do Norte, Abatedouros, epidemiologia, frequência.

Abstract

This dissertation is made up of two chapters. In the first chapter, the objective was to determine the occurrence of tuberculosis by *Mycobacterium bovis* in cattle slaughtered in two public slaughterhouses of the Central Potiguar mesorregion, state of Rio Grande do Norte, Brazil. In the period from November 2017 to August 2018, 11616 carcasses had a routine inspection, where lesions suggestive of tuberculosis were observed in one animal (0.009%). Samples of tissues located from the lung, heart and spleen were submitted to bacteriological culture and molecular diagnosis. The omentum was used for histopathological examination and Ziehl-Neelsen staining. The lesions were cultured for 90 days in Stonebrink medium, but there was no growth. When nested-PCR was used, lung, heart and spleen samples were positive. The histopathological exam of the omentum showed the presence of multiple granulomas of necrotic center and areas of mineralization and when it was submitted to the staining of Ziehl-Neelsen, the presence of acid-fast bacilli was detected. Microbiological culture, despite being considered gold standard, may present false negative results, besides being a time-consuming technique, making it difficult to use for surveillance in slaughterhouses. The presence of a positive animal in the study represents a serious public health problem, especially for breeders, scavengers, technicians and the end consumer. Nested-PCR and histopathology present fast and effective results for the diagnosis of the disease. Therefore, one of the essential systems applied to the control of bovine tuberculosis is the epidemiological surveillance of animals in slaughterhouses. The objective of chapter II was to determine the frequency of positive animals for bovine tuberculosis in the state of Rio Grande do Norte. The data were provided by the Institute of Defense and Agricultural Inspection of Rio Grande do Norte (IDIARN) and come from its Local Animal and Plant Health Units (ULSAV'S) collected from the monthly reports issued by veterinarians authorized to work under the National Program of the Control and Eradication of Brucellosis and of Animal Tuberculosis (PNCEBT), covering the period from June 2012 to June 2018. For the diagnosis, the simple cervical test and the cervical comparative test were used as the screening test. In total, 16889 cattle were tested, of which 44 animals (0,26%) presented a positive result. There was no significant difference ($P < 0,05$) in the frequency of positivity between females (0,25%) and males (1,16%). In view of this result and considering the importance of local bovine farming to the economy, it is important to conduct measures that include producers' awareness, sanitary control in the acquisition and sale of matrices, inspection of sanitary barriers and periodic surveys of the epidemiological situation of this disease, especially in the units with the highest frequency of positive animals, with the aim of avoiding, or at least minimizing, the spread of the agent.

Keywords: Tuberculosis, *Mycobacterium bovis*, Rio Grande do Norte, Slaughterhouses, epidemiology, frequency.

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Lista de abreviaturas, siglas e símbolos

%-Porcentagem

°C-Grau Celsius

µl-Microlitro

µM-Micromolar

BAAR-Bacilo Álcool-Ácido resistente

CEP-Comitê de Ética em Pesquisa

cm-Centímetro

CSTR-Centro de Saúde e Tecnologia Rural

DNA-Ácido Desoxirribonucleico

dNTP-Base nitrogenada (A, C, G ou T)

EMBRAPA-Empresa Brasileira de Pesquisa Agropecuária

GTA-Guia de Trânsito Animal

IDIARN-Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte

KCl-Cloreto de Potássio

LAVADI-Laboratório de Vacinas e Diagnóstico

LPA-Laboratório de Anatomia Patológica/histopatológica

M. bovis-Mycobacterium bovis

MAPA-Ministério da Agricultura, Pecuária e Abastecimento

$MgCl_2$-Cloreto de Magnésio

Mg-Miligrama

ml-Mililitro

mM-Milimolar

MS-Mato Grosso do Sul

OMS-Organização Mundial de Saúde

PB- Paraíba

PCR-Reação em Cadeia da Polimerase

PE-Pernambuco

pH-Potencial de Hidrogênio

pmol-Picomol

PNCEBT-Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal

RN-Rio Grande do Norte

Rpm-Rotação por Minuto

SDS-Dodecil Sulfato de Sódio

SIM-Serviço de Inspeção Municipal

Taq-*Thermus aquaticus*

TCC-Teste Cervical Comparativo

TCS-Teste Cervical Simples

TE-Tampão TRIS-EDTA

TRIS-Tris (hidroximetil) aminometano

UFCG-Universidade Federal de Campina Grande

UFPB-Universidade Federal da Paraíba

ULSAV-Unidade Local de Sanidade Animal e Vegetal

1 Introdução Geral

A bovinocultura constitui-se em uma atividade econômica e social de elevada importância para o estado do Rio Grande do Norte, contribuindo para a criação de empregos e geração de renda. Porém, fatores relacionados a baixa tecnificação na atividade por parte dos produtores, a ocorrência de uma ampla e intensa seca nos últimos anos e a presença de doenças infecciosas têm causado prejuízos significativos aos criadores de animais no Estado (SEPLAN, 2016).

Dentre essas patologias, destaca-se a tuberculose bovina, doença zoonótica causada pelo *Mycobacterium bovis*. A enfermidade tem um impacto econômico significativo, reduzindo a produção de carne e leite e impedindo o uso de carcaças e partes afetadas que não são adequadas para o consumo humano (FAO.; OMS.; OIE., 2017).

Desde 2006, a Organização Mundial de Saúde (OMS) estabelece como paradigma para o combate às zoonoses a necessidade de cooperação entre as medicinas veterinária e humana, elaborando em conjunto pesquisas no campo da epidemiologia, bem como trabalhando novas ferramentas para diagnóstico e vigilância das doenças que acometem os seres vivos de modo geral (SILVA et al., 2018).

No Brasil, o Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (PNCEBT), instituído no ano de 2001 e recentemente atualizado pela Instrução Normativa nº10/2017, tem como objetivo reduzir a prevalência e a incidência de brucelose e de tuberculose bovina e bubalina, visando sua erradicação (BRASIL, 2017). Este regulamento preconiza o abate

sanitário de bovinos positivos no teste intradérmico de tuberculinização no diagnóstico *ante-mortem* e a inspeção de carcaças com lesões macroscópicas em abatedouros no *post-mortem* (ARAÚJO et al., 2014).

Estudos realizados em diversos Estados do nordeste do país têm demonstrado a prevalência da doença. Bahiense et al. (2016), realizaram inquérito epidemiológico para caracterizar a situação da enfermidade na Bahia, sendo observado uma prevalência de focos de 1,6% e 0,21% a de animais. No estado de Pernambuco, Lima et al. (2016), determinaram como fatores de risco da enfermidade a criação de 18 ou mais vacas no rebanho, ordenhar as vacas 2 ou 3 vezes ao dia e ao fato dos animais compartilharem pastagem. No presente estudo, a prevalência de focos foi de 2,87% e 0,62% a de animais.

É necessário conhecer a real condição sanitária do rebanho bovino do estado do Rio Grande do Norte, para que medidas de controle possam ser estabelecidas, visando a erradicação da enfermidade. Desta forma, o capítulo I desta dissertação, teve como objetivo determinar a ocorrência de tuberculose por *Mycobacterium bovis* em bovinos abatidos em dois abatedouros públicos da mesorregião Central Potiguar.

Devido a esta necessidade, o capítulo II teve como objetivo determinar a frequência de animais positivos para a tuberculose bovina no estado do Rio Grande do Norte, através de dados fornecidos pelo Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte (IDIARN).

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3 capítulo I

Tuberculosis in cattle slaughtered in the Central Potiguar mesoregion, State of Rio Grande do Norte, Brazil (Tuberculose em bovinos abatidos na mesorregião Central Potiguar, estado do Rio Grande do Norte)

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Tuberculosis in cattle slaughtered in the Central Potiguar mesoregion, state of Rio Grande do Norte, Brazil

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ABSTRACT

The objective of the study was to determine the occurrence of tuberculosis in bovine animals slaughtered in two public slaughterhouses of the Central Potiguar mesorregion, state of Rio Grande do Norte. Between November 2017 and August 2018, a total of 11,616 bovine carcasses underwent routine *post-mortem* inspection, with lesions suggestive of tuberculosis in one animal (0.009%). Samples of tissues located in the lung,

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heart and spleen were submitted to bacteriological culture and molecular diagnosis. The omentum was used for histopathological examination and Ziehl-Neelsen staining. The lesions were cultured for 90 days in *Stonebrink* medium, but there was no growth. When nested-PCR was used, the lung, heart and spleen samples were positive. The histopathological examination of the omentum showed the presence of multiple granulomas of necrotic center and areas of mineralization and when they were submitted to Ziehl-Neelsen staining, it was detected the presence of alcohol-acid resistant bacilli (BAAR). In this way, it can be verified that the microbiological culture, despite being considered gold standard, can present false negative results, besides being a time-consuming technique, making it difficult to use for surveillance in slaughterhouses. The presence of a positive animal in the study represents a problem regarding public health, especially for breeders, slaughterhouse workers, technicians and the end consumer. Nested-PCR and histopathology present fast and effective results for the diagnosis of the disease. Therefore, one of the essential systems applied to the control of bovine tuberculosis is the epidemiological surveillance of animals in slaughterhouses.

KEYWORDS bovine tuberculosis - *Mycobacterium bovis* - slaughterhouses - food safety.

INTRODUCTION

The bovine farming constitutes an economic and social activity of great importance for the state of Rio Grande do Norte, contributing for the creation of jobs and generation of income. However, factors related to the low technification of the activity on the part of the producers, the occurrence of a wide and intense drought in the last years

and the presence of infectious diseases have caused significant damages to the breeders of animals in the State (SEPLAN, 2016).

Infectious diseases include tuberculosis, which is a zoonotic infection caused by *Mycobacterium bovis*, the primary host of which is cattle, but several domestic and wild mammal species, including humans, are also susceptible (Carvalho *et al*., 2015). In cattle, tuberculosis presents a chronic evolution and is characterized by granulomatous lesions located predominantly in the respiratory tract (Bica, Copetti and Brum, 2018). In humans, the main route of transmission of *M. bovis* is indirect, occurring generally by the consumption of milk and other contaminated dairy products not subjected to heat treatment or by the consumption of raw or undercooked contaminated meat (FAO., OMS. and OIE., 2017).

In Brazil, the National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis (PNCEBT) was instituted in 2001 and updated in 2017 by Normative Instruction nº10/2017 (Brasil, 2017). After 15 years of its creation, the epidemiological situation of bovine tuberculosis was detailed in 13 states of the federation, but the state of Rio Grande do Norte had no data published in the study (Neto *et al.*, 2016).

Of the control measures recommended by the PNCEBT, sanitary inspection in slaughterhouses is of great importance for public health, since it promotes the removal of meat contaminated with the pathogenic microorganisms, since uncoated animal carcasses end up going into clandestine commerce and serving as food for the population (Ramos *et al.,* 2016).

Several studies carried out in slaughterhouses in Brazil have shown the occurrence of disease in animals. Filho *et al.* (2014) identified the occurrence of tuberculosis in cattle

by *M. bovis* through lesions observed in carcasses during routine *post-mortem* inspection in slaughterhouses in the state of Bahia. In a similar study carried out at the Garanhuns-Pernambuco public slaughterhouse, the occurrence of tuberculosis in cattle by *M. bovis* was also determined from lesions collected during the inspection (Silva *et al.,* 2018a). However, the state of Rio Grande do Norte has no official data on the actual health status of the herd, which has contributed to the devaluation of the local cattle.

Thus, the present work aimed to determine the occurrence of *Mycobacterium bovis* tuberculosis in cattle slaughtered in public slaughterhouses of the Central Potiguar mesorregion, state of Rio Grande do Norte, Brazil.

MATERIAL AND METHODS

STUDY AREA

The present study was carried out in two slaughterhouses of the State, holders of the Municipal Inspection Service (SIM), located in the municipality of Caicó (latitude: 6º 27 '30 "S; longitude: 37º 05' 52" W) and Jardim do Seridó (latitude: 6º 35 '04 "S, longitude: 36º 46' 28" W), Central Potiguar mesoregion, Northeast of Brazil.

SAMPLING

In the period from November 2017 to August 2018, all bovine animals that were slaughtered underwent routine inspection. After entry into the slaughterhouses, the cattle had their Animal Transit Guide (ATG) checked and were sent to the arrival pens for the

ante-mortem examination. In the post-mortem, all the carcasses were inspected in the different lines, having their main organs and lymph nodes incised. The carcass that presented lesions suggestive of tuberculosis had samples collected, packaged in sterile, hermetically sealed flasks, duly identified and sent in an ice cube box to the Laboratory of Vaccines and Diagnosis at the Center for Health and Rural Technology of the Federal University of Campina Grande, Campus in Patos-Paraíba (LAVADI/CSTR/UFCG), where, under biosecurity conditions, they were subdivided into duplicates and frozen. Subsequently, an aliquot was sent to the Immunology Laboratory, located at the Embrapa Beef Cattle, Campo Grande-Mato Grosso do Sul, to perform the microbiological and molecular diagnosis, and the second part was fixed in 10% formaldehyde and sent for histopathological examination in the Laboratory of Pathological/Histopathological Anatomy from the UFCG (LPA/CSTR/UFCG).

MICROBIOLOGICAL CULTURE

The lesions suggestive of tuberculosis were thawed and minced with the aid of a scalpel in smaller sizes, placed in tubes containing ceramic beads (MagNA Lyser green beads tube). 1ml of sterile distilled water was added and the mixture was stirred in the MagNA Lyser instrument (Roche, Mannheim, Germany) for 30 seconds at 6.000rpm. The aqueous fraction was removed, transferred to sterile and decontaminated 2ml tubes using the Petroff method (Petroff, 1915). Cultivation was prepared by instilling 6 drops of each inoculum in duplicate into the tubes with *Stonebrink* medium and incubated at 37°C in a bacteriological oven being monitored for 90 days.

MOLECULAR ANALYSIS

Molecular analysis of lesions suggestive of tuberculosis

DNA extraction

DNA extraction from the lesions was performed using the DNEasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the protocol recommended by the manufacturer. The quality and concentration of the DNA samples were evaluated by spectrophotometry on NanoDrop ND-2000 (Thermo Scientific, Wilmington, USA) (Araújo *et al.,* 2014).

Nested-PCR for the TbD1 region

According to Araújo *et al.*, (2014), this technique aims at the amplification of the TbD1 region using a first amplification of the genomic region by conventional PCR, followed by amplification of the product of the first one (re-amplification), using real-time PCR by nested-PCR. This region comprises the *mmpS6* gene, which codes for a probable conserved membrane protein, and the 5 'region of the *mmpL6* gene. TbD1 is present in *M. bovis* (including BCG strains), *Mycobacterium africanum*, *M. canettii* and absent in modern *M. tuberculosis* isolates. Conventional PCR (step 1) was performed in a final volume of 25µl, containing 10mM Tris-HCl (pH 8.3), 50µM KCl; 1.5mM MgCl2, 0.2mM of each dNTP, 7.5pmol of each *primer*, 1.25U of *Taq* DNA polymerase and 3.0µl of DNA. Amplifications were performed on a MJ Mini Thermal Cycler thermal cycler (BIO-RAD, California, USA). Initial denaturation was performed at 95°C for 4 minutes, followed by 35 cycles of denaturation at 95°C for 90 seconds, annealing at 65°C with 35

cycles for 30 seconds and extension at 72°C for 45 seconds. A single final extension step of 72°C was performed for 3 minutes. The external *forward primers* Mb: 5'-GTGGCGGTCGCGGGATTCAGCGTCTAT-3' and outer *reverse* Mb: 5'-TTATGGCGGCCACACCCACCCAAAACAG-3' were used, which amplify a 474bp fragment of the TbD1 region.

The real-time PCR (step 2) was performed in final volume of 12.5µl, containing 6.25µl of Taqman Master Mix (ref. 4352042, Applied Biosystems, California, USA), 600nM of each *primer*, 100nM probe and 3µl of the standard PCR product. Amplifications were performed on a StepOnePlus thermal cycler (Applied Biosystems, California, USA). Initial denaturation was performed with one cycle at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 62°C for 30 seconds. In this reaction, the internal *forward primers* Mb: 5'-GCGGTCTTCGCCAATGTT-3' and internal *reverse* Mb: 5'-GCAGCCGATGGAATTGCT-3'; and the probe Mb: 6FAM-CGCGCAAGGCGA-MGBNFQ were used, which amplify a fragment of 51bp.

For all nested-PCR reactions, a positive control was used with *M. bovis* AN5 DNA, a negative control with a *M. tuberculosis H37Rv* sample, and a blank control containing all the reagents of the reaction mix, minus the DNA that is replaced by equal volume of ultrapure water. It is valid to postulate that, in order to avoid contamination of the preparation of the mix and the pipetting of DNA, the whole process took place inside a specific cabinet for PCR.

Molecular analysis of colonies

Extraction of bacterial DNA by thermolysis

The cultures had aliquots transferred to 1.5ml microtubes, where 200µl TE buffer was added and incubated for 60 minutes at 87.5°C for inactivation of the colonies. After the inactivation process, 200µl of bacterial suspension was used in the DNA extraction process. Sixty microliters of lysozyme (10mg/ml) were added to the samples. The mixture was incubated for 120 minutes at 37°C. In order to break down the proteins, 30ml of Proteinase K (10 mg/ml) and 60ml of 10% SDS were added to the samples. Samples were incubated at 56°C for 240 minutes. Four hundred microliters of saturated phenol pH 8.0 and 15µl of isoamyl alcohol were used to remove the proteins from the solution. Each sample was centrifuged at 700rpm for 5 minutes at environmental temperature. The supernatant (aqueous phase) was removed and transferred to a new tube. DNA precipitation was performed using 275µl of absolute ethanol and 15µl of 2-propanol. The samples were centrifuged at 19.000rpm for 10 minutes at 4°C. After discarding the supernatant, the precipitate was washed with 500µl of 70% ethanol and the tube was homogenized by inversion. The sample was again centrifuged at 19.000rpm for 10 minutes at 4°C and the supernatant was discarded. The DNA was dried and resuspended in 50µl TE buffer (Sales *et al.,* 2014).

Conventional PCR reaction

The standard PCR reaction of the colonies was performed in a final volume of 25µl containing 10mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 0.2mM of each dNTP, 7.5pmol of each *primer*, 1.25U of *Taq* DNA polymerase and 2.0µl of DNA. Amplifications were performed on a MJ Mini Thermal Cycler (BIO-RAD, California, USA). Initial denaturation was performed at 95°C for 4 minutes, followed by 35 cycles

of denaturation at 95°C for 30 seconds, annealing at 56°C with 35 cycles for 30 seconds and extension at 72°C for 30 seconds. A single final extension step of 72°C was performed for 5 minutes. Outer *forward primers* Mb.400: 5'-AACGCGACGACCTCATATTC-3' and outer *reverse* Mb.400: 5'-AAGGCGAACAGATTCAGCAT-3' were used. Amplified PCR products were all subjected to 2% agarose gel electrophoresis stained with ethidium bromide (0.5μg/ml) (Araújo *et al.,* 2014).

RESULTS

During the period of collection, a total of 11,616 bovine carcasses underwent routine *post-mortem* inspection, with lesions suggestive of tuberculosis (Fig 1) in one animal (0.009%). The animal came from a dairy farm of the municipality of Caicó, created in the semi extensive system, it was an adult female that had a good body score. The lesions were mainly located in organs and tissues of the thoracic and abdominal cavities. The lesions in the lung were characterized by firm, whitish and encapsulated nodules, 2-3cm in diameter and filled with caseous material. In the heart, calcified multiple lesions and in variable sizes could be observed. In the spleen, a number of granulomatous nodules with a purulent or caseous aspect were found, with a fibrous capsule and, in some cases, calcification in the center of the lesion, as evidenced by the knife grinding at the cut. In the omentum, multiple granulomas, yellowed, well delimited and firm, were observed in the *post-mortem* inspection, which, when cut, were filled with caseous material and with knife grinding at the cut.

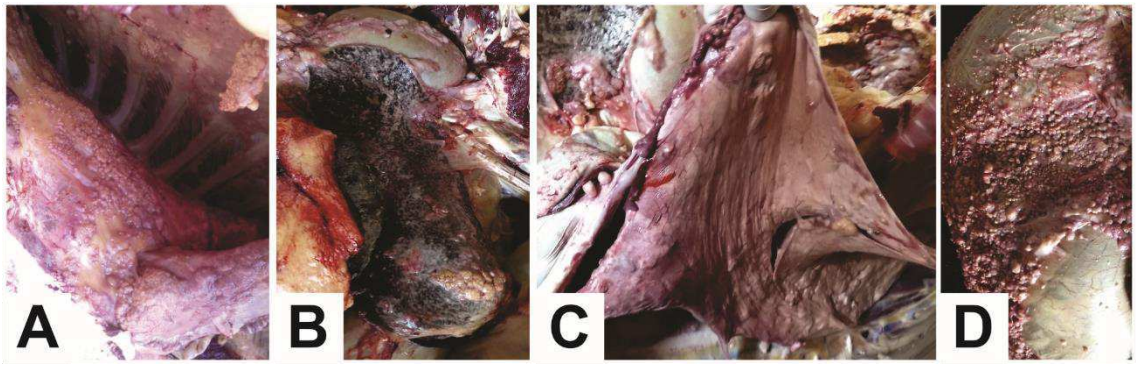

Fig 1 Tuberculosis in cattle (A) Bovine carcass condemned with lesions suggestive of bovine tuberculosis (B) Spleen with caseous nodules (C) Lung presenting innumerable firm nodules (D) Presence of multiple, yellowish and firm granulomas in the omentum

In the histopathological exam of the omentum, we observed multiple granulomas, characterized by multifocal areas with central necrosis coalescents surrounded by a marked mononuclear inflammatory infiltrate, predominantly composed of macrophages, epithelioid macrophages, multinucleated giant cells and, to a lesser extent, plasma cells and lymphocytes, coated by a thick capsule of fibrous connective tissue (Fig 2). In some areas of necrosis, deposition of strongly basophilic granular material (mineralization) was observed. In the staining of Ziehl-Neelsen, the presence of alcohol-acid resistant bacilli (BAAR) was detected in the cytoplasm of macrophages and in the middle of necrosis.

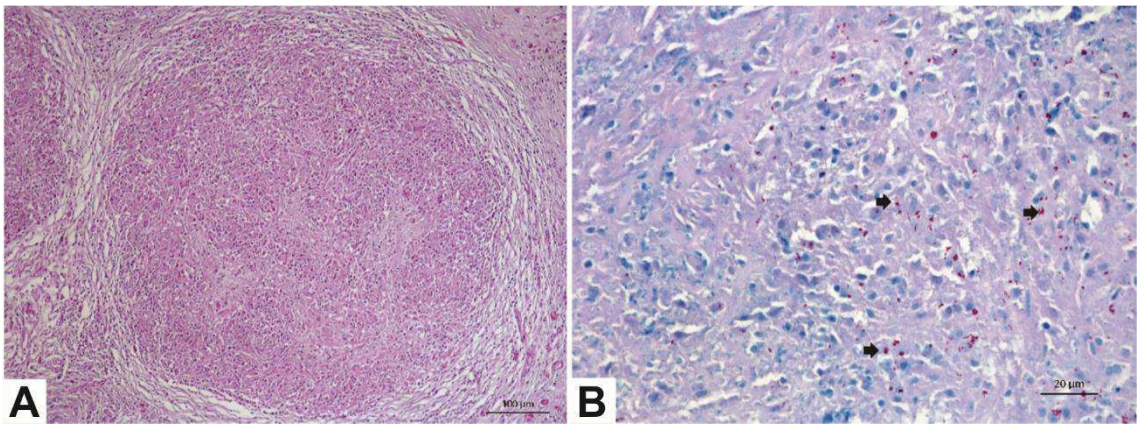

Fig 2 Tuberculosis in cattle (A) Omentum, granuloma is seen consisting of a central area of necrosis associated with a marked inflammatory infiltrate consisting of macrophages, multinucleated giant cells, epithelioid cells and plasma cells surrounded by fibrous connective tissue HE, Bar=100µm (B) Omentum, Multiple alcohol-acid resistant bacilli (arrow) Ziehl-Neelsen, Bar=20µm

The lung, heart and spleen samples, analyzed by nested-PCR, were considered positive, since they exhibited amplification for the TbD1 region of *M. bovis* (Fig 3). Regarding the culture in *Stonebrink* medium, two samples (heart and spleen) evidenced growth of colonies, but were not confirmed as being of *M. bovis* by conventional PCR. The tissue samples presented agreement with positive results, both in histopathology and nested-PCR.

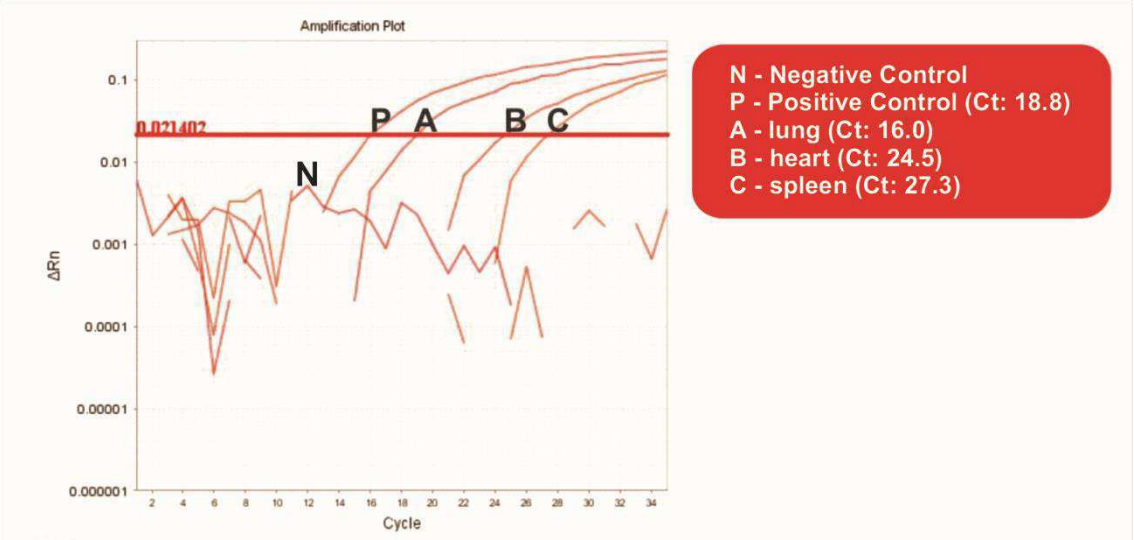

Fig 3 Samples showing nested-PCR amplification for *Mycobacterium bovis* TbD1

DISCUSSION

The animals slaughtered in the establishments studied come from all the mesoregion in which they are situated. The presence of animals tested positive for

tuberculosis represents a risk from the point of view of public health, since the ingestion of contaminated meat is the cause of several diseases that will cost directly to the health systems and indirectly to the work capacity.

Carvalho *et al.* (2015), in a study carried out with cattle slaughtered in Mato Grosso, found that of the 41,193 carcasses examined, 198 (0.48%) had suspected tuberculosis lesions. Silva *et al.* (2018a), in a work carried out in the Garanhuns-Pernambuco public slaughterhouse, observed that of the 3,180 carcasses that were inspected, 32 (1%) presented lesions indicative of tuberculosis in the *post-mortem* examination, presenting a caseous and calcified aspect.

The low frequency found in the study was already expected, since the state of Rio Grande do Norte is facing the occurrence of an intense drought in recent years, which caused a significant reduction in the cattle herd, due also to the fact that animals slaughtered are mostly young. Araújo *et al.* (2014) and Pereira *et al.* (2017), report the chronicity of the disease, being observed, in most cases, in adult animals. It is also important to highlight the active surveillance by the PNCEBT in the State, supervised by the Institute of Defense and Agricultural Inspection of Rio Grande do Norte (IDIARN), which has contributed to reduce the prevalence and incidence of bovine tuberculosis (Brasil, 2017).

Regarding the distribution of lesions in the carcass, the presence in several organs and tissues of the thoracic and abdominal cavities was found. Data similar to those observed by Filho *et al.* (2014), who inspected 825,394 carcasses in ten slaughterhouses in Bahia, found that the lesions were located mainly in the lymph nodes of the head and neck, as well as in the thoracic, lung, liver and peritoneal lymph nodes.

The lesions found in this research are reported to be the most common form of disease observed at *post-mortem* inspection in cattle (Bica, Copetti and Brum, 2018). In a similar study carried out by Ramos *et al.* (2016), at the *post-mortem* inspection, lesions suggestive of tuberculosis were characterized by focal, multifocal or diffuse nodules, with a caseous appearance and a gritty cut surface in various organs and tissues. In these, the identification of *M. bovis* could be confirmed with histopathological examination, mycobacterial culture, Ziehl-Neelsen staining and molecular diagnosis.

In the present study, one of the methods used to diagnose the lesions was nested-PCR. In order to amplify the TbD1 region (conserved part of *M. bovis*), all samples submitted to the test showed positive results. In a study carried out by Araújo *et al.* (2014), in Campo Grande-Mato Grosso do Sul, with lesions collected from bovines and buffaloes in several slaughterhouses of the country, it was observed that nested-PCR allowed the identification of *M. bovis* in tissues with similar or superior performance to the culture and in a short time. Furlanetto *et al.* (2012), using multiple PCR directly on tissue fragments, detected the presence of *M. bovis* in 7% (14/182) of the lesions, demonstrating greater sensitivity of the molecular test when compared to the microbiological analysis (1.5%).

The inspection performed by the traditional method in this research, through the incision of the main organs and lymph nodes, may represent a risk to the health of the official veterinarian, the carcass and the environment. On the basis of this evidence, the European Commission published the regulation 219/2014. With this regulation, *post-mortem* inspection in domestic pigs was only visual, and incision and palpation of the carcass and offal were performed when signs and clinical lesions could indicate a possible risk (Ghidini et al., 2018).

Silva *et al.* (2018b), evaluating the risk factors of zoonotic tuberculosis in Brazil, associated the cases found to occupational exposure and consumption of milk and non-pasteurized derivatives, since they put individuals in direct and indirect contact with the animals and their excretions/secretions. In research carried out in Argentina, it was observed that the cases of human tuberculosis caused by *M. bovis*, have shown a tendency to decline after the implantation of improvements in food hygiene and the pasteurization of milk and its derivatives (Kantor, LoBue and Thoen, 2010).

In establishments where the occurrence of the disease is low, identification of infected animals becomes even more difficult. At present, there is no diagnostic method, *ante* or *post-mortem*, capable of identifying all infected animals. Detection is improved when different diagnostic methods such as molecular, microbiological and histopathological are combined.

CONCLUSION

The presence of a positive animal in the study represents a problem regarding public health, especially for breeders, slaughtermen, technicians and the end consumer. Microbiological culture, despite being considered gold standard, may present false negative results, besides being a slow technique. Nested-PCR and histopathology proved to be quite useful, demonstrating rapid and effective results in the diagnosis of the disease. Therefore, one of the essential systems applied to the control of bovine tuberculosis is the epidemiological surveillance of animals in slaughterhouses.

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COMPLIANCE WITH ETHICAL STANDARDS

Ethical approval This research was approved by the Research Ethics Committee (CEP) from the Federal University of Campina Grande (UFCG) under number 107/2017.

Conflict of interest statement Authors declare no conflict of interest.

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4 capítulo II

Tuberculose bovina no estado do Rio Grande do Norte: estudo retrospectivo

Manuscrito submetido à revista Pesquisa Veterinária Brasileira.

Qualis B2 para Zootecnia / Recursos Pesqueiros.

Tuberculose bovina no estado do Rio Grande do Norte: estudo retrospectivo[2]

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ABSTRACT.-Neto P. S. B., Medeiros G. B., Gameleira J. A. L., Maia R. D., Rodrigues O. G., Medeiros R. S., Dantas A. F. M., Azevedo S. S. & Higino S. S. S. 2019. **Tuberculose bovina no estado do Rio Grande do Norte: estudo retrospectivo.** Pesquisa Veterinária Brasileira 00(0):00-00. Programa de Pós-Graduação em Ciência Animal, Centro de Saúde e Tecnologia Rural, Universidade Federal de Campina Grande, Avenida Universitária, s/n, Santa Cecília, Patos, PB 58708-110, Brasil. E-mail: higinosss@gmail.com

The objective of the present study was to determine the frequency of positive animals for bovine tuberculosis in the state of Rio Grande do Norte, Brazil. The data were provided by the Institute of Defense and Agricultural Inspection of Rio Grande do Norte (IDIARN) and come from its Local Animal and Plant Health Units (ULSAV'S) collected from the monthly reports issued by veterinarians authorized to work under the National Program of the Control and Eradication of Brucellosis and of Animal Tuberculosis (PNCEBT), covering the period from June 2012 to June 2018. For the diagnosis, the simple cervical test and the cervical comparative test were used as the screening test. In total, 16.889 cattle were tested, of which 44 animals (0,26%) presented a positive result. There was no significant difference (P <0,05) in the frequency of positivity between females (0,25%) and males (1,16%). In view of this result and considering the importance of local bovine farming to the economy, it is important to conduct measures that include producers' awareness, sanitary control in the acquisition and sale of matrices, inspection of sanitary barriers and periodic surveys of the epidemiological situation of this disease, especially in the Units with the highest frequency of positive animals, with the aim of avoiding, or at least minimizing, the spread of the agent.

INDEX TERMS: *Mycobacterium bovis,* tuberculosis, epidemiology, frequency.

RESUMO.-[Tuberculose bovina no estado do Rio Grande do Norte: estudo retrospectivo.] O objetivo do presente trabalho foi determinar a frequência de animais positivos para a tuberculose bovina no estado do Rio Grande do Norte, Brasil. Os dados foram fornecidos pelo Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte (IDIARN) e são oriundos de suas Unidades Locais de Sanidade Animal e Vegetal (ULSAV'S) coletados dos relatórios mensais emitidos por médicos veterinários habilitados para atuação no âmbito do Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (PNCEBT), compreendendo o período de junho de 2012 a junho de 2018. Para o diagnóstico foi utilizada, como prova de triagem, o teste cervical simples e como prova confirmatória o teste cervical comparativo. No total, foram testados 16.889 bovinos, dos quais 44 animais (0,26%) apresentaram resultado positivo. Não foi observada diferença significativa (P < 0,05) na frequência de positividade entre fêmeas (0,25%) e machos (1,16%). Frente a este resultado e considerando a importância da bovinocultura local para a economia, é importante a condução de medidas que incluam a conscientização dos produtores, controle sanitário na aquisição e venda de matrizes, fiscalização nas barreiras sanitárias e levantamentos periódicos da situação epidemiológica desta doença, principalmente nas Unidades com maior frequência de animais positivos, com o objetivo de evitar, ou pelo menos minimizar, a disseminação do agente.

TERMOS DE INDEXAÇÃO: *Mycobacterium bovis*, tuberculose, epidemiologia, frequência.

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INTRODUÇÃO

A economia do estado do Rio Grande do Norte (RN) baseia-se na agricultura, pecuária, indústria, turismo e serviços. Dentro desta, o gado bovino ocupa um papel importante pois contribui para a criação de empregos e geração de renda. Contudo, essa atividade ainda apresenta algumas limitações. Nos últimos anos, o ocorrência de uma ampla e intensa seca, além da baixa tecnificação e a existência de doenças infecciosas, têm causado prejuízos significativos aos criadores no Estado (SEPLAN 2016).

Dentre essas patologias, destaca-se a tuberculose bovina, causada pelo *Mycobacterium bovis*. O microrganismo é adaptado ao gado como hospedeiro, mas também causa a enfermidade em outras espécies animais incluindo o homem (CESAR et al. 2016). A doença tem um impacto econômico significativo, reduzindo a produção de carne e leite e impedindo o uso de carcaças e partes afetadas que não são adequadas para o consumo humano (FAO, OMS & OIE 2017).

No Brasil, o Programa Nacional de Controle e Erradicação da Brucelose e da Tuberculose Animal (PNCEBT), instituído no ano de 2001 e recentemente atualizado pela Instrução Normativa nº10/2017, tem como objetivo reduzir a prevalência e a incidência de brucelose e de tuberculose bovina e bubalina, visando sua erradicação (BRASIL 2017). Este regulamento preconiza o abate sanitário de bovinos positivos no teste intradérmico de tuberculinização no diagnóstico *ante-mortem* e a inspeção de carcaças com lesões macroscópicas em abatedouros no *post-mortem* (ARAÚJO et al. 2014).

Diversos estudos tem sido conduzidos com o objetivo de caracterizar a situação epidemiológica da tuberculose bovina nas Unidades Federativas do Brasil (BAHIENSE et al. 2016, BELCHIOR et al. 2016, DIAS et al. 2016, GALVIS et al. 2016, GUEDES et al. 2016, LIMA et al. 2016, NÉSPOLI et al. 2016, QUEIROZ et al. 2016, RIBEIRO et al. 2016, ROCHA et al. 2016, SILVA et al. 2016, VELOSO et al. 2016 & VENDRAME et al. 2016). No entanto, o estado do Rio Grande do Norte não dispõe de dados oficiais sobre a real condição sanitária do rebanho, o que tem contribuído para a desvalorização da pecuária local.

Considerando a importância do PNCEBT na garantia da cadeia produtiva da carne bovina e do leite e a inexistência de dados sobre a situação epidemiológica da tuberculose bovina no Estado, o presente estudo teve como objetivo determinar a frequência de animais positivos para a tuberculose bovina no Rio Grande do Norte, no período de junho de 2012 a junho de 2018.

MATERIAL E MÉTODOS

O estado do Rio Grande do Norte possui 167 municípios e, segundo o Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte (IDIARN), estão divididos em 12 Unidades Locais de Sanidade Animal e Vegetal (ULSAV'S), a saber: Unidade de Assú, Caicó, Currais Novos, João Câmara, Mossoró, Nova Cruz, Umarizal, Santa Cruz, São Paulo do Potengi, Parnamirim, Pau dos Ferros e Lajes (Fig.1).

Foram utilizados dados fornecidos pelo IDIARN decorrentes dos relatórios estaduais mensais emitidos por médicos veterinários habilitados para atuação no âmbito do PNCEBT pelo Ministério da Agricultura, Pecuária e Abastecimento (MAPA) das diferentes ULSAV'S. Os dados utilizados compreenderam o período de junho de 2012 a junho de 2018.

Para o diagnóstico da infecção por *Mycobacterium bovis* foi utilizado o teste cervical simples (TCS), utilizado como teste de rotina e o teste cervical comparativo (TSC) para o diagnóstico confirmatório, observando as condições e critérios que são estabelecidos pelo PNCEBT (BRASIL 2017).

Para o cálculo das frequências de animais positivos por unidades, foi utilizado o programa Microsoft® Excel 2013. Já para a frequência de animais positivos por sexo, foi utilizado o teste G com correção de continuidade de Yates e nível de significância de 5%, através do programa BioEstat versão 5.03 (AYRES et al. 2007).

RESULTADOS E DISCUSSÃO

Dos 16.889 bovinos testados, 44 animais (0,26%) apresentaram resultado positivo na tuberculinização. Das 16.717 fêmeas, 42 (0,25%) foram positivas, enquanto dos 172 machos, 2 (1,16%) foram positivos (**Quadro 1**). Não foi observada diferença significativa na frequência de positividade entre fêmeas e machos (P = 0,213).

A figura 2 apresenta a distribuição da frequência de animais positivos por unidades no estado Rio Grande do Norte, no período de junho de 2012 a junho de 2018.

Nesta pesquisa verificou-se uma baixa frequência de animais positivos, onde 44 (0,26%) bovinos foram positivos para tuberculose. Dados similares foram observados no estado da Paraíba, onde 54.472 bovinos foram testados e 136 (0,25%) foram positivos para a enfermidade (FIGUEIREDO et al. 2010). Resultados superiores foram observados em uma pesquisa realizada no estado do Rio Grande do Sul, onde foram testados 62.149 animais e detectou-se uma frequência de 0,87% (TODESCHINI et al. 2018).

Ao que pese as diferenças entre os modelos de estudos aplicados, em uma pesquisa realizada no estado da Bahia visando caracterizar a situação da enfermidade, foi possível determinar uma prevalência de 0,21% (BAHIENSE et al. 2016). Já no estado de Pernambuco, Lima et al. (2016) determinaram uma prevalência de animais positivos de 0,62%.

No estado do Rio Grande do Norte, a criação de bovinos é predominantemente de rebanhos leiteiros, porém 79% dos estabelecimentos caracterizam-se como de agricultura familiar, onde são produzidos 44% da produção estadual de leite bovino. Geralmente, a atividade leiteira praticada por este modelo de criação, se caracteriza pelo baixo nível tecnológico, adotando sistemas de produção menos intensivos, com pouco uso de capital, produção diversificada e uso de recursos disponíveis na própria unidade produtiva (SEPLAN 2016). Em estudo realizado por Neto et al. (2016) foi observado os maiores índices de prevalência de rebanhos infectados e de bovinos positivos ao teste tuberculínico no estado do Espírito Santo, norte de São Paulo, sul de Minas Gerais e sul de Goiás, coincidindo com o cinturão produtor de leite no Brasil.

Fatores externos como o clima e a ocorrência de uma intensa seca nos últimos anos podem ter contribuído para minorar a disseminação da doença no território do Rio Grande do Norte. De fato, o clima quente do estado do Rio Grande do Norte e a maior incidência de raios solares com dois períodos distintos (inverno curto e longa estação seca), particularmente na região semiárida, podem diminuir a permanência e viabilidade do agente no meio ambiente. O Estado apresentou uma redução no efetivo de bovinos nos últimos anos, passando de 907.185 em 2006 para 757.945 em 2017 (IBGE 2017).

Analisando a distribuição da ocorrência da tuberculose no estado do Rio Grande do Norte, verificou-se maiores frequências de positividade em animais nas ULSAV'S de Currais Novos, Santa Cruz e Caicó (1,52%, 0,52% e 0,50%, respectivamente). É importante destacar que estas unidades juntas apresentam 28% do efetivo de bovino do Estado (IBGE 2017) o que poderia explicar o maior número de animais positivos.

CONCLUSÃO

Considerando a importância da bovinocultura local para a economia, é importante a condução de medidas que incluam a conscientização dos produtores, controle sanitário na aquisição e venda de matrizes, fiscalização nas barreiras sanitárias e levantamentos periódicos da situação epidemiológica desta doença, principalmente nas ULSAV's com maior frequência de animais positivos, com o objetivo de evitar, ou pelo menos minimizar, a disseminação do agente.

Agradecimentos - Ao Instituto de Defesa e Inspeção Agropecuária do Rio Grande do Norte, por ter disponibilizado os dados.

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Legendas das Figuras

Fig.1. Unidades Locais de Sanidade Animal e Vegetal (ULSAV'S) e seus municípios.

Fig.2. Distribuição de animais positivos para a tuberculose bovina, nas 12 ULSAV'S do estado do Rio Grande do Norte, de junho de 2012 a junho de 2018.

Legenda do quadro

Quadro 1. Animais positivos para tuberculose bovina, nas 12 ULSAV'S do estado do Rio Grande do Norte, no período de junho de 2012 a junho de 2018.

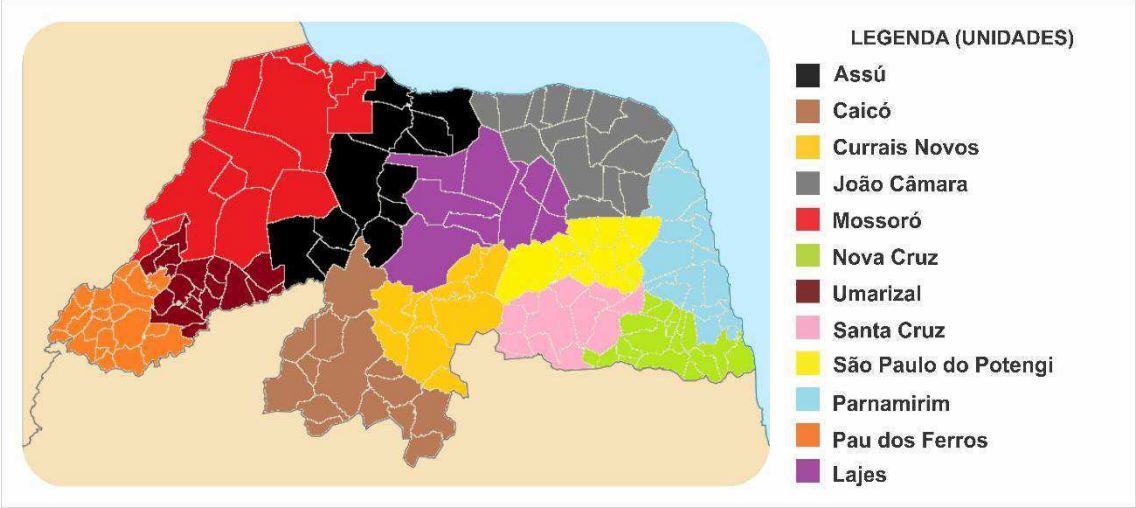

LEGENDA (UNIDADES)
Assú
Caicó
Currais Novos
João Câmara
Mossoró
Nova Cruz
Umarizal
Santa Cruz
São Paulo do Potengi
Parnamirim
Pau dos Ferros
Lajes

Fig.1.

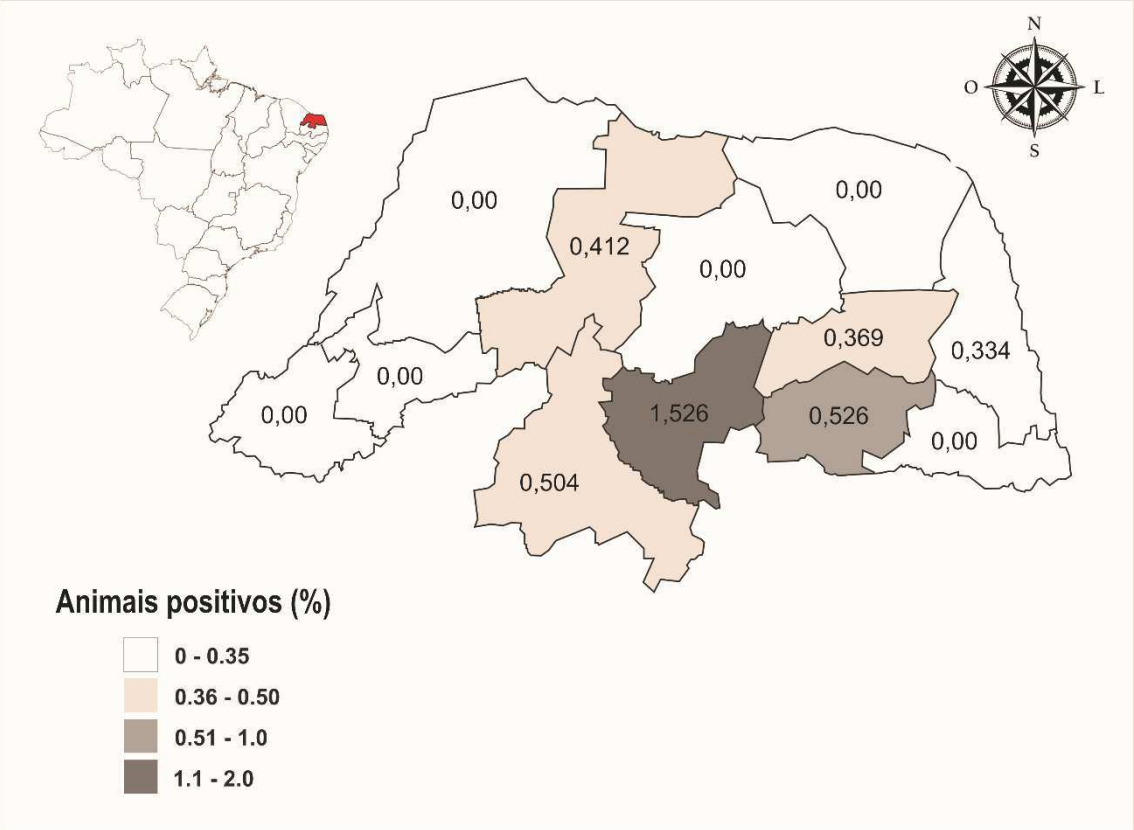

N
O
L
S
0,00
0,412
0,00
0,00
0,00
0,00
0,369
0,334
1,526
0,526
0,00
0,504
Animais positivos (%)
0 - 0.35
0.36 - 0.50
0.51 - 1.0
1.1 - 2.0

Fig.2.

ULSAV'S	TOTAL POR UNIDADE						ANIMAIS POSITIVOS					
	MACHOS	%	FÊMEAS	%	TOTAL	%	MACHOS	%	FÊMEAS	%	TOTAL	%
ASSÚ	12	0,990099	1200	99,0099	1212	7,176269	1	8,333333	4	0,333333	5	0,412541
CAICÓ	22	1,232493	1763	98,76751	1785	10,56901	0	0,00	9	0,510493	9	0,504202
CURRAIS NOVOS	12	1,308615	905	98,69138	917	5,42957	1	8,333333	13	1,436464	14	1,526718
JOÃO CÂMARA	18	1,628959	1087	98,37104	1105	6,54272	0	0,00	0	0,00	0	0,00
MOSSORÓ	21	1,118807	1856	98,88119	1877	11,11374	0	0,00	0	0,00	0	0,00
NOVA CRUZ	0	0,00	311	100	311	1,841435	0	0,00	0	0,00	0	0,00
UMARIAZAL	38	1,110137	3385	98,88986	3423	20,26763	0	0,00	0	0,00	0	0,00
SANTA CRUZ	9	1,578947	561	98,42105	570	3,374978	0	0,00	3	0,534759	3	0,526316
SÃO PAULO DO POTENGI	16	0,843437	1881	99,15656	1897	11,23216	0	0,00	7	0,372142	7	0,369004
PARNAMIRIM	13	0,724638	1781	99,27536	1794	10,6223	0	0,00	6	0,336889	6	0,334448
PAU DOS FERROS	7	0,4851	1436	99,5149	1443	8,544023	0	0,00	0	0,00	0	0,00
LAJES	4	0,720721	551	99,27928	555	3,286163	0	0,00	0	0,00	0	0,00
TOTAL	**172**	**1,018414**	**16717**	**98,98159**	**16889**	**100**	**2**	**1,162791**	**42**	**0,251241**	**44**	**0,260525**

Quadro1.

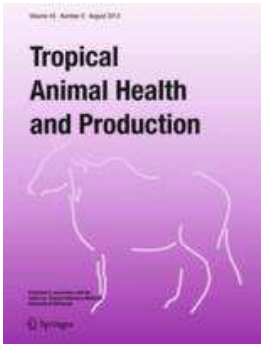
5 Conclusões Gerais

Diante da importância da bovinocultura para a economia do estado do Rio Grande do Norte, a presença de animais positivos para tuberculose, verificados nesta pesquisa, representa um sério problema no que diz respeito à saúde pública, principalmente para criadores, magarefes, técnicos e para o consumidor final.

Os métodos de diagnósticos utilizados neste estudo, *ante-mortem* (tuberculinização) e *post-mortem* (Cultivo microbiológico, histopatológico e molecular) contribuíram na identificação dos animais positivos.

É importante a condução de medidas mais abrangentes que incluam a conscientização dos produtores, controle sanitário na aquisição e venda de matrizes, fiscalização nas barreiras sanitárias e em abatedouros e levantamentos periódicos da situação epidemiológica desta doença, com o objetivo de evitar, ou pelo menos minimizar, a disseminação do agente e o impacto causado por este.

Anexos



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LaTeX macro package (zip, 182 kB)

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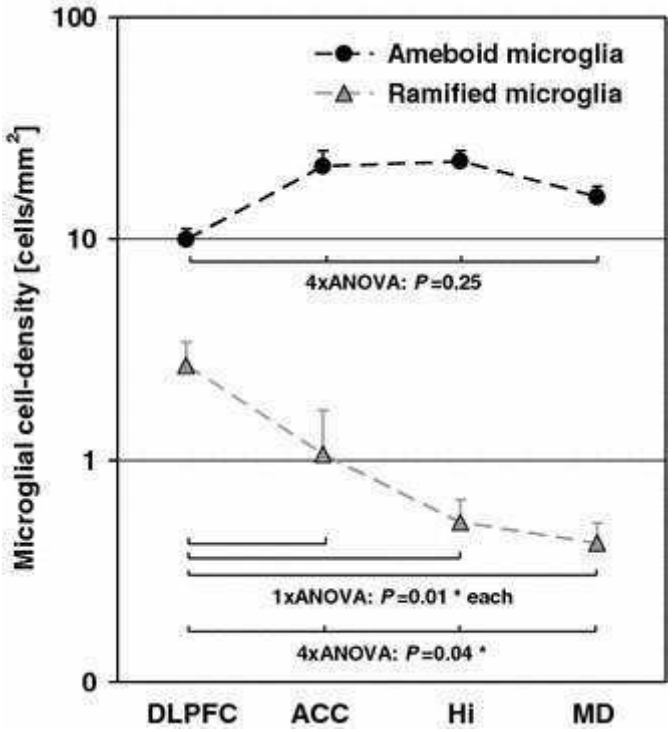

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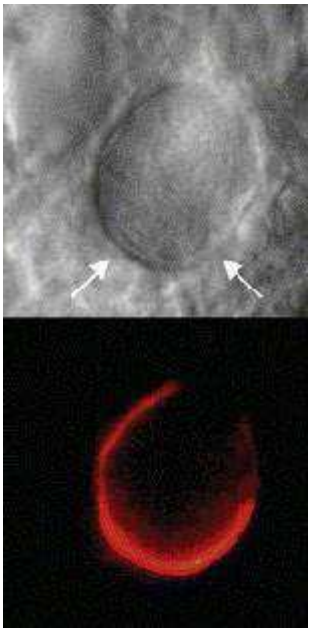

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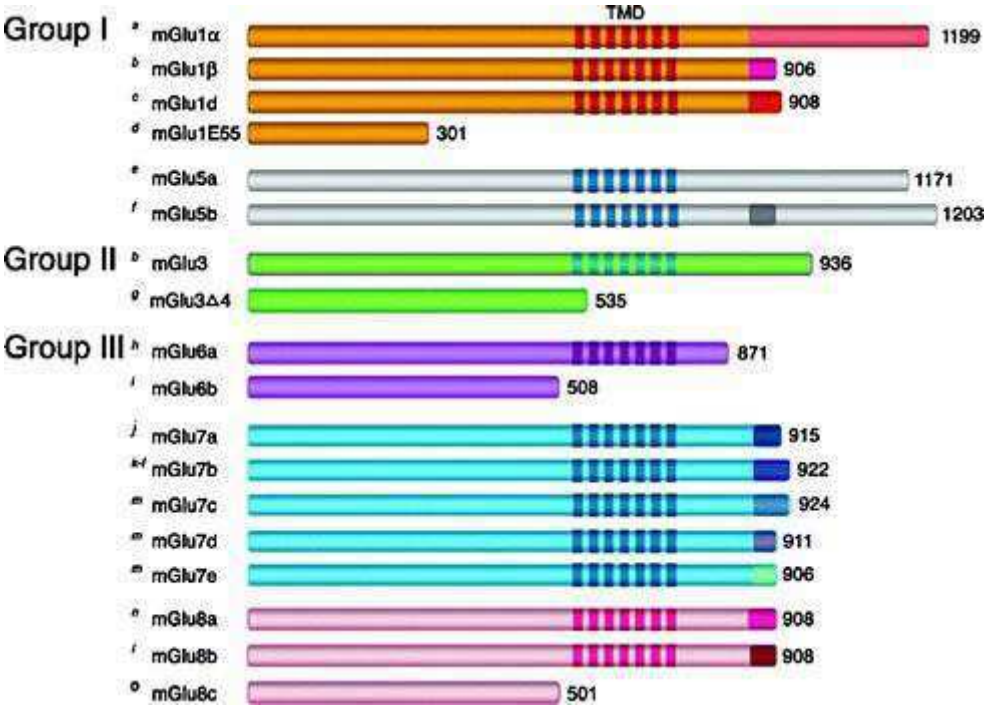

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Combination artwork should have a minimum resolution of 600 dpi.

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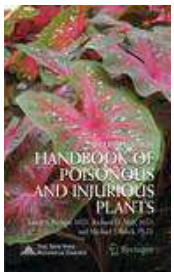

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d) O **Rodapé da primeira página** deve conter os endereços profissionais postais completos dos autores (evitando-se traços horizontais), na língua do país do respectivo autor (em português, espanhol, inglês) e seus e-mails; o e-mail do autor para 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 têrmos 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 marcgravii*), no início de cada capítulo (**TÍTULO, ABSTRACT, RESUMO, INTRODUÇÃO, etc.**), quando aparecem pela primeira vez, seguido da abreviação do gênero (p.ex. *P. marcgravii*).

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; de conveniência quando seguida de letra alta (35 kg ou 35kg, 4 h ou 4h). A abreviação de número é "nº" e não "nº"; grau Celsius é "ºC" e não "ºC".

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, Armién 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 parêntees 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.

3. **Apresentação das Figuras:**

a) As figuras devem ser salvas em 300dpi, arquivo TIF.
b) Enviar cada figura separadamente.
c) Identificar as figuras em ordem conforme a menção no texto.
d) As figuras solitárias devem ter seus arquivos identificados como (Fig.1, Fig.2 ...)

e) 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.

f) 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.

g) 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.).

h) 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!)

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