UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

CARACTERIZAÇÃO PATOLÓGICA E MICROBIOLÓGICA DE LESÕES NA GLÂNDULA MAMÁRIA DE VACAS LEITEIRAS NO SUL DO BRASIL

Ronaldo Michel Bianchi

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Autor: Me. Ronaldo Michel Bianchi

Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias da Universidade Federal do Rio Grande do Sul na área de concentração em Medicina Veterinária Preventiva e Patologia: Patologia Animal e Patologia Clínica

Orientador: Prof. Dr. David Driemeier

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AGRADECIMENTOS

RESUMO

Lesões na glândula mamária de bovinos leiteiros são de grande importância, pois geram grande impacto à cadeia produtiva leiteira. Devido a isso, nessa tese estão incluídos dois artigos científicos acerca do tema, com destaque para as mastites e a papilomatose de tetos. O primeiro trabalho objetivou caracterizar os achados macroscópicos e histológicos de mastites em vacas leiteiras e correlacioná-las com os patógenos envolvidos. Para isso, amostras de leite e fragmentos de tecido de cada quarto mamário de vacas leiteiras abatidas foram encaminhados para análise microbiológica e histopatológica, respectivamente. No total, 148 vacas e 592 quartos mamários foram coletados. Desses, 432 (73%) apresentaram lesões inflamatórias (mastite), classificadas em sete padrões de acordo com a análise histopatológica. Os padrões misto, linfoplasmocítico e supurativo foram os mais prevalentes com 35,9% (155/432), 27,1% (117/432) e 14,3% (62/432) dos casos, respectivamente, e associaram-se aos mesmos patógenos: *Streptococcus* spp., *Staphylococcus* coagulase negativa (SCN), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis* e *Corynebacterium bovis*. Lesões piogranulomatosas foram observadas em 7,2% (31/432) dos casos, com diferentes padrões de acordo com o agente envolvido, principalmente ocasionadas por *S. aureus* e *Nocardia* sp. Mastite abscedativa representou 6,0% (26/432) dos casos, predominantemente associada a *Trueperella pyogenes*. O padrão necrossupurativo foi observado em 5,8% (25/432) dos casos, associado a bactérias como SCN e *Escherichia coli*. Mastite granulomatosa representou apenas 3,7% (16/432) dos casos e foi ocasionalmente associada ao *Mycobacterium* sp. O segundo estudo teve por objetivo descrever os aspectos moleculares e patológicos de papilomas em tetos de 73 vacas leiteiras encaminhadas ao abate. Fragmentos das lesões foram coletados em *pools* individuais por animal e submetidas à análise molecular. Os tetos com as lesões remanescentes processados e submetidos à análise histopatológica. Os papilomas apresentaram três padrões macroscópicos: exofítico (5 [6,9%]), plano (29 [39,7%]) e misto (39 [53,4%]). Histologicamente, todas as amostras foram identificadas como papilomas escamosos. Na análise molecular, em 27 amostras foram identificados oito tipos clássicos de papilomavírus bovino (BPVs 4, 6, 7, 8, 9, 10, 11 e 12); em 17 amostras, seis prováveis tipos de BPV previamente descritos; e em 15 amostras, 10 prováveis novos tipos de BPV.

Palavras-chave: bovinos leiteiros, doenças infecciosas, patologia mamária, mastite, papilomatose de tetos, microbiologia.

ABSTRACT

Lesions in the mammary gland of dairy cattle are very important once they have a big impact on dairy industry. Thus, two scientific articles on this subject, especially mastitis and teat papillomatosis, are included in this thesis. The first research aimed to characterize the gross and microscopic features of mastitis in dairy cows, and to correlate them with the pathogens involved. For this, milk samples and tissue fragments from each mammary quarter of slaughtered dairy cows were sent for microbiological and histopathological analysis, respectively. A total of 148 cows and 592 mammary quarters were collected. From these, 432 quarters (73%) had inflammatory lesions (mastitis) that were classified into seven patterns based on the histopathological findings. Mixed, lymphoplasmacytic and suppurative patterns were the most prevalent with 35.9% (155/432), 27.1% (117/432) and 14.3% (62/432) of the cases, respectively, and they were associated with the same set of pathogens: *Streptococcus spp.*, coagulase-negative *Staphylococcus* (CNS), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis* and *Corynebacterium bovis*. Pyogranulomatous lesions were observed in 7.2% (31/432) of the cases with distinct patterns based on the agent involved, mostly *S. aureus* and *Nocardia sp.* Abscedative mastitis accounted for 6.0% (26/432) of the cases, predominantly associated with *Trueperella pyogenes*. The necrosuppurative pattern was observed in 5.8% (25/432) of the cases, and it was associated with bacteria such as CNS and *Escherichia coli*. Granulomatous mastitis represented only 3.7% (16/432) of the cases, and it was occasionally associated with *Mycobacterium sp.* The second research aimed to describe the molecular and pathological aspects of teat papillomas in 73 slaughtered dairy cows. Fragments of the lesions were collected in individual pools per animal and were subjected to molecular analysis. The teats with the remaining lesions were processed and subjected to histopathological analysis. Papillomas presented three macroscopic patterns: exophytic (5 [6.9%]), flat (29 [39.7%]) and mixed (39 [53.4%]). Histologically, all samples were identified as squamous papillomas. Based on the molecular analysis, eight classical types of bovine papillomavirus (BPVs 4, 6, 7, 8, 9, 10, 11 and 12) were identified in 27 samples; six previously reported putative BPV types in 17 samples; and 10 putative new BPV types in 15 samples.

Keywords: dairy cattle, infectious diseases, mammary pathology, mastitis, teat papillomatosis, microbiology.

SUMÁRIO

1. INTRODUÇÃO

O Brasil é um dos maiores produtores mundiais de leite com 33,5 bilhões de litros produzidos em 2017. Dentre as regiões produtoras do país, há destaque para a região Sul, que ocupa a primeira posição no ranking da produção nacional de leite desde 2015, quando ultrapassou a região Sudeste. Em 2017, a região Sul foi responsável por 35,7% da produção nacional, com média de 3284 litros/vaca/ano, bem superior à média nacional de 1963 litros/vaca/ano (IBGE, 2018).

Embora o Brasil seja um grande produtor mundial de leite, produzir um produto de qualidade ainda constitui um desafio. A qualidade e a quantidade do leite podem ser influenciadas por diferentes fatores, tais como: o processo de obtenção, armazenamento e transporte, por fatores zootécnicos relacionados ao manejo, alimentação e genética dos bovinos, assim como por fatores sanitários da glândula mamária e do bovino (PAUL; GANGULY, 2014; ACOSTA et al., 2016). Desta forma, a mastite, que consiste no processo inflamatório da glândula mamária, é uma das doenças mais importantes e um grave problema sanitário para bovinos leiteiros, pois acomete rebanhos por todo mundo e gera grandes prejuízos à cadeia produtiva leiteira, tanto pela diminuição no volume e na qualidade do leite produzido, quanto pelos gastos com tratamentos, descarte de leite, morte e descarte precoce de animais (BRADLEY, 2002; BANDEIRA et al., 2013; BUSANELLO et al., 2017).

A glândula mamária é uma unidade secretória composta por alvéolos arranjados em lóbulos, por ductos e sinus lactíferos, pelas cisternas da glândula e do teto, além do ducto papilar (canal do teto). O ducto papilar termina em um óstio, que é circundado por um esfíncter (SCHLAFER; FOSTER, 2016). A glândula mamária possui mecanismos de defesa inatos e adaptativos que impedem a entrada ou combatem patógenos no seu interior. Estes mecanismos incluem a estrutura do ducto papilar, o acúmulo de queratina no esfíncter do teto, o fluxo do leite pelo canal do teto, a presença de fatores solúveis no leite, como lactoferrinas, lisozimas, complemento e citocinas, além de fatores celulares e humorais (OVIEDO-BOYSO et al., 2007; FOSTER, 2017).

Quando esses mecanismos de defesa são ultrapassados, as mastites podem ocorrer e são causadas principalmente por bactérias (BANDEIRA et al., 2013; MARKEY et al., 2013; SCHLAFER; FOSTER, 2016; FOSTER, 2017). A principal porta de entrada na glândula mamária é de forma ascendente, através do óstio e ducto papilar (BENITES et al., 2002; OVIEDO-BOYSO et al., 2007; ARANTES, 2014). Entretanto, há formas menos importantes,

como a via hematógena, em casos de tuberculose, brucelose e micoplasmose, ou por meio de lesão penetrante (SANTOS; NASCIMENTO; EDWARDS, 2016; FOSTER, 2017). Além disso, fatores mecânicos associados à ordenha também podem induzir danos ao ducto papilar quando o processo e o equipamento não estiverem bem regulados. Esses fatores incluem principalmente vácuo excessivo, sobreordenha e má pulsação, e podem provocar a eversão parcial do ducto, hiperqueratose, ulceração e fibrose, facilitando a entrada de micro-organismos (ARANTES, 2014; SANTOS; NASCIMENTO; EDWARDS, 2016; SCHLAFER; FOSTER, 2016).

Apesar de as mastites serem causadas tipicamente por bactérias através da infecção ascendente da glândula mamária, diferentes formas de apresentação da doença podem ser influenciadas pela resposta do hospedeiro, pela patogenicidade do micro-organismo envolvido e por fatores ambientais (BENITES et al., 2002; SCHLAFER; FOSTER, 2016; FOSTER, 2017). Todos esses fatores podem provocar alterações físicas, químicas e microbiológicas no leite produzido, assim como alterações na glândula mamária, que servem como critérios para diagnóstico da doença (ZHAO; LACASSE, 2008; AKERS; NICKERSON, 2011). A mastite apresenta-se na forma clínica, quando são evidentes os sinais da inflamação, como rubor, aumento de volume, edema e aumento de sensibilidade ao toque no quarto mamário afetado, além da presença de grumos no leite; e subclínica, na qual são necessários testes de campo como o *California Mastitis Test* (CMT) para diagnóstico da condição (BANDEIRA et al., 2013; ACOSTA et al., 2016).

Quanto aos patógenos mamários, esses podem ser divididos em contagiosos e ambientais, de acordo com seu habitat e fonte de infecção (BRADLEY, 2002; OVIEDO-BOYSO et al., 2007; FOSTER, 2017). O primeiro grupo (contagiosos) tem a glândula mamária como principal local de persistência ou reservatório. Tais agentes são transmitidos de uma vaca para outra, principalmente no momento da ordenha, e incluem bactérias como *Streptococcus agalactiae* e *Staphylococcus aureus* (FERREIRA et al., 2006; MARKEY et al., 2013; FOSTER, 2017). O segundo grupo (ambientais) inclui patógenos encontrados no solo, nas fezes, na água e no alimento, e a transmissão ocorre no período entre as ordenhas. Esses incluem bactérias como *Escherichia coli*, *Klebsiella* spp. e *Nocardia* spp, além de fungos, como *Cryptococcus neoformans*, e algas, como *Prototheca* spp. (BRADLEY, 2002; SCHLAFER; FOSTER, 2016; FOSTER, 2017).

Lesões papilomatosas nos tetos e/ou no úbere também são frequentemente descritas em rebanhos leiteiros (MAEDA et al., 2007; TOZATO et al., 2013; SILVA et al., 2015). São

causadas por vários tipos de papilomavírus bovino (BPVs), que são vírus envelopados, DNA fita dupla, pertencentes à família Papillomaviridae, e acometem principalmente animais jovens (de VILLIERS et al., 2004; MAEDA et al., 2007; HATAMA et al., 2009; ALFIERI et al., 2012; TOZATO et al., 2013; ARANTES, 2014; SILVA et al., 2015).

Em vacas leiteiras a papilomatose de tetos pode resultar em grandes prejuízos à sanidade e à estrutura da glândula mamária. Os papilomas podem ser grandes o suficiente para dificultar a limpeza dos tetos, além de causar interferência no processo de ordenha e fluxo do leite, especialmente quando localizados próximos ao esfíncter do teto, predispondo a ocorrência de mastites. Também, a ulceração e ruptura das lesões podem provocar sangramentos e distorção dos ductos lactíferos (CAMPO, 2003; GEORGE et al., 2008; TOZATO et al., 2013; BOCANETI et al., 2016).

Macroscopicamente, diferentes padrões de papilomatose podem ser observados em diferentes regiões anatômicas, que incluem os tetos (MAEDA et al., 2007; BATISTA et al., 2013; TOZATO et al., 2013; SILVA et al., 2015; MAULDIN; PETERS-KENNEDY, 2016). Entretanto, histologicamente, dois tipos de papiloma podem ser identificados dependendo do tipo de BPV envolvido. BPVs do gênero *Xipapillomavirus* são classicamente epiteliotrópicos restritos, portanto induzem a formação de papilomas escamosos, também conhecidos como papilomas verdadeiros. Já BPVs do gênero *Deltapapillomavirus* infectam tanto a epiderme quanto a derme e, portanto, induzem a formação de fibropapilomas (de VILLIERS et al., 2004; MAEDA et al., 2007; MAULDIN; PETERS-KENNEDY, 2016).

Os papilomavírus necessitam de uma diferenciação celular do epitélio para seu desenvolvimento, portanto o isolamento e a amplificação em sistemas *in vitro* de cultivos celulares não podem ser realizados (ALFIERI et al., 2012). Desta forma, são utilizadas técnicas para o diagnóstico baseadas na identificação viral, como a reação em cadeia da polimerase (PCR) por meio de *primers* degenerados (FAP59/FAP64). Essa técnica amplifica fragmentos parciais da porção mais conservada do gene L1 do BPV, que seguida da amplificação do produto, permitem a identificação de diferentes tipos de BPV em bovinos (de VILLIERS et al., 2004; OGAWA et al., 2004; CLAUS et al., 2008; LUNARDI et al., 2013; TOZATO et al., 2013; SILVA et al., 2015).

Embora o Brasil seja um dos maiores produtores mundiais de leite, com destaque para a região Sul do país, diferentes fatores podem interferir na quantidade e na qualidade do leite produzido, assim como no perfil sanitário e rentabilidade do rebanho. Dentre esses, destacam-se as lesões na glândula mamária. Dessa forma, esse estudo tem como objetivos: (1) realizar

uma análise patológica e microbiológica das lesões na glândula mamária de vacas leiteiras abatidas no Sul do Brasil; e (2) caracterizar os diferentes padrões macroscópicos e histológicos de mastite e papilomatose em tetos e correlacioná-los aos agentes patogênicos identificados.

2. ARTIGO 1

Nesse item é apresentado o artigo intitulado:

Pathological and microbiological characterization of mastitis in dairy cows

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4   Pathological and microbiological characterization of mastitis in dairy cows

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11   **Abstract.**

12   Mastitis may be caused by a wide range of microorganisms able to induce distinct lesions in mammary tissues.

13   This study aims to characterize the gross and microscopic features of mastitis in dairy cows and to correlate them

14   with the pathogens involved. The udders of slaughtered dairy cows were inspected and milk samples from each

15   mammary quarter or samples of the parenchyma were sent for microbiological analysis, and tissue collected for

16   histopathological evaluation. A total of 148 cows and 592 mammary quarters were collected. From these, 432

17   quarters (73%) had mastitis and in 160 (27%) no changes were observed. Mastitis was classified into seven patterns

18   based on the histopathological findings, of which mixed, lymphoplasmacytic and suppurative mastitis were the

19   most prevalent with 35.9% (155/432), 27.1% (117/432) and 14.3% (62/432) of the cases, respectively. These

20   patterns were associated with the same set of pathogens: *Streptococcus* spp., coagulase-negative *Staphylococcus*

21   (CNS), *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus uberis* and *Corynebacterium bovis*. The

22   pyogranulomatous pattern represented 7.2% (31/432) of the cases with distinct distribution based on the agent

23   involved, mostly *S. aureus* and *Nocardia* sp. Abscedative mastitis accounted for 6.0% (26/432) of the cases; it was

24   characterized by multiple abscesses in the parenchyma, and was mainly caused by *Trueperella pyogenes*.

25   Necrosuppurative mastitis represented 5.8% (25/432) of the cases, which were characterized by severe

26   parenchyma necrosis, and were caused by bacteria such as CNS and *Escherichia coli*. The granulomatous pattern

27   represented 3.7% (16/432) of the cases, and was occasionally associated with *Mycobacterium* sp.

28   **Keywords:** mammary gland; dairy cattle; mammary pathology; bacteria.

29

30   **Introduction**

31   Mastitis is an important disease of dairy cattle and represents a challenge for the dairy industry since it

32   causes losses associated with the reduction of production and quality of milk, treatment expenses, milk discard,

33   and cattle mortality (Hazlett et al, 1984; Bradley 2002; Acosta et al. 2016; Busanello et al. 2017). The disease is

34   often caused by contagious or environmental pathogenic microorganisms (Oviedo-Boyso et al. 2007), mainly

35   bacteria (Bandeira et al. 2013; Foster 2017), capable of inducing various lesions in mammary tissues (Zhao and

36   Lacasse, 2008; Akers and Nickerson 2011). Studies that performed an evaluation correlating these pathological

37   lesions with the pathogenic agents causing mastitis are scarce (Macadam 1958; Hazlett et al. 1984; Benites et al.

38   2002; Hussian et al. 2012). Surveys are usually conducted to determine the frequency of the major agents involved

39   in mastitis cases (Bandeira et al. 2013; Cunha et al. 2015; Acosta et al. 2016; Busanello et al. 2017); in addition to

40   studies on the description of case reports or outbreaks related to a single microorganism (Schiefer et al. 1976;

41   Shibahara and Nakamura 1999; Pisoni et al. 2008; Tessele et al. 2014). Therefore, this work aims to characterize

42   the pathological aspects of inflammatory lesions in the mammary glands of slaughtered dairy cows in the Southern

43   region of Brazil and determine their correlations with the pathogens involved.

44

45   **Materials and Methods**

46   From August of 2016 to March of 2017, the slaughter of dairy cows was carried out in two

47   slaughterhouses located in the state of Rio Grande do Sul, Brazil. The mammary glands were randomly selected

48   and inspected. After inspection, milk samples were collected by manual milking using aseptic technique from each

49   mammary quarter and kept under refrigeration in sterile tubes. In cases where it was not possible to obtain the milk

50   sample, a fragment of the mammary parenchyma was collected. After that, fragments of mammary tissue were

51   collected and kept in 10% neutral buffered formalin.

52   All collected samples, either 0.01 ml of milk or a loopful of parenchyma, were cultured in both 5% Sheep

53   Blood Agar (Kasvi®) and MacConkey Agar (Kasvi®). Plates were incubated in a CO^2 enriched atmosphere (~5%)

54   at 37°C and examined after 24, 48 and 72 hours. Bacterial species were identified by their cultural, morphological,

55   tinctorial and biochemical characteristics using a simplified scheme based on National Mastitis Council manual

56   guidelines (1999). Hemolytic, catalase-positive, coagulase-positive, Maltose-positive and Mannitol-positive

57   Gram-positive cocci in cell clusters were classified as *Staphylococcus aureus*. Remaining isolates of coagulase-

58   negative, catalase-positive Gram-positive cocci were grouped as "coagulase-negative *Staphylococcus*" (CNS).

59   Catalase negative, Gram-positive cocci arranged in pairs or chains were identified as *Streptococci*. Based on the

60   results for CAMP reaction and esculin hydrolysis the isolates were presumptively identified as *Streptococcus*

61   *agalactiae* (CAMP+, Esculin-), *S. uberis* (CAMP+/-, Esculin+) and *S. dysgalactiae* (CAMP-, Esculin-).

62   Complementary fermentation tests of Inulin, Lactose, Mannitol, Raffinose, Salicin, Sorbitol and, Trehalose

63   (MARKEY et al. 2013a), either confirmed the preliminary identification or when biochemical discrepancies were

64   found, reclassified an isolate as *Streptococcus* spp. Straight, catalase positive, large-sized Gram-positive rods from

65   strongly hemolytic colonies were classified as *Bacillus* sp. Irregular shaped, lipophilic, Gram-positive rods were

66   classified as *Corynebacterium bovis*. Slow growing (48h or more), hemolytic pin-point colonies, highly proteolytic

67   (on Loeffler's medium slants) Gram-positive cocci or irregular rods were deemed as *Trueperella pyogenes*. Slow-

68   growing (>72h), white, powdery, firmly adherent to the medium colonies displaying branching Gram-labile

69   filaments on microscopy were identified as *Nocardia* sp. Gram-negative rods were tested for catalase, oxidase,

70   and Lactose fermentation on MacConkey Agar and then more comprehensively characterized using API20E strips

71 (BioMerieux, Marcy l'Étoile, France). In cases where the simplified identification scheme was unable to result in

72 a proper species discrimination, additional tests were conducted following Markey et al. (2013a).

73   The formalin-fixed material was routinely processed for histopathology and stained by hematoxylin and

74 eosin (H&E). Histopathological analysis was carried out, and the inflammatory alterations were classified

75 according to their morphological aspect in mixed, lymphoplasmacytic, suppurative, pyogranulomatous,

76 abscedative, necrosuppurative, and granulomatous mastitis. In cases in which granulomatous and

77 pyogranulomatous lesions were observed without the identification of agents by microbiological examination, the

78 histochemical techniques of Grocott Methenamine Silver and Ziehl-Neelsen (ZN) were performed. Additionally,

79 sections of mammary parenchyma with pyogranulomatous or necrossupurative lesions suggestive of *Nocardia* sp.

80 were submitted to immunohistochemistry (IHC). A polyclonal anti-*Nocardia* spp. antibody (non-commercial) was

81 used at a dilution of 1:50. Amplification signal was achieved by using the Mack 4 Universal HRP polymer (Biocare

82 Medical®) and the reaction was revealed with the 3-amino-9-ethyl-carbazole chromogen (Biocare Medical®).

83 Furthermore, a section of one mammary quarter was submitted to IHC for *Fusobacterium necrophorum* with a

84 polyclonal antibody produced in rabbit (strain ATCC25286) (Shibahara et al. 2002).

85

86 **Results**

87   A total of 148 mammary glands were collected and 592 mammary quarters were analyzed. Of these, 432

88 (73%) showed inflammatory lesions and in 160 (27%) no changes were observed. However, when the distribution

89 of the inflammatory lesions in the mammary gland of each cow was analyzed, it was verified that 5.4% (8/148) of

90 the cows presented mastitis in one quarter, 13.5% (20/148) in two quarters, 24.3% (36/148) in three quarters,

91 46.6% (69/148) in all quarters, and 10.2% (15/148) had no inflammatory lesions.

92   The inflammatory lesions (mastitis) observed in the mammary quarters were morphologically classified

93 into mixed (35.9% [155/432]); lymphoplasmacytic (27.1% [117/432]), suppurative (14.3% [62/432]),

94 pyogranulomatous (7.2% [31/432]), abscedative (6.0% [26/432]), necrosuppurative (5.8% [25/432]) and

95 granulomatous (3.7% [16/432]). The agents identified in the lesions are listed in the Table 1 and were subdivided

96 according to the histological pattern of the associated mastitis. Of the 432 bacteriological cultures, correlated to

97 the mammary quarters with mastitis, pure and mixed cultures, were isolated, respectively, in 45.8% (198/432) and

98 in 14.8% (64/432), while in 39.4% (170/432) no significant bacterial growth (<10 CFU [<103/ml]) or no bacterial

99 growth was detected.

100   Grossly, the mixed mastitis was characterized by the pronounced lobular mammary pattern, with small

101   yellowish nodules (0.2-0.5 cm in diameter) in the middle of the parenchyma and projecting towards the lumen of

102   the ducts and gland cistern, which were interspersed by thin white septa (Figure 1A). The histological pattern was

103   constituted of a discrete to moderate inflammatory infiltrate, composed of neutrophils within the alveoli and ducts

104   as well as a multifocal infiltrate of neutrophils, lymphocytes, plasma cells and macrophages in the interstitium. It

105   was commonly associated with hyperplasia and degeneration of epithelial cells and discrete fibrosis (Figure 1B).

106   Occasionally, bacterial myriads, squamous metaplasia of the glandular epithelium, alveolar dilatation and

107   formation of fibrous polyps in the ductal lumina were observed. *Streptococcus* spp., coagulase-negative

108   *Staphylococcus* (CNS), *Staphylococcus aureus* and *Corynebacterium bovis* were the main agents identified in pure

109   or mixed cultures, associated with each other.

110   Suppurative mastitis presented a gross pattern similar to that described for mixed mastitis (Figure 1C).

111   Histologically, mild to moderate amounts of intact and degenerate neutrophils were observed in the alveoli, ducts

112   and the interstitium. They were often associated with hyperplasia and degeneration of epithelial cells (Figure 1D).

113   Bacterial myriads were occasionally visualized in association with the inflammatory infiltrate, as well as squamous

114   metaplasia of the glandular epithelium, alveolar dilatation and fibrosis. *Streptococcus* spp. and CNS were the main

115   bacterial agents isolated, either pure or mixed cultures, mainly in association with *C. bovis*.

116   Grossly, the lymphoplasmacytic pattern was characterized by firm mammary quarters with a decrease of

117   mammary lobulations, and thick white septa dissecting the parenchyma (Figure 2A). Histopathologically, it

118   consisted of discrete to moderate interstitial inflammatory infiltrate composed of lymphocytes and plasma cells,

119   with occasional macrophages, associated with moderate fibrosis (Figure 2B). Sometimes, nodular, white, and

120   polypoid structures (Figure 2C) were observed macroscopically, which in histology corresponded to dilated alveoli

121   covered by hyperplastic epithelium (Figure 2D). In this category, the main microorganisms isolated in pure or

122   mixed culture and associated with each other, were *Streptococcus* spp., CNS and, *C. bovis*.

123   In the pyogranulomatous mastitis, three bacteria were identified in pure cultures (*S. aureus*, *Pseudomonas*

124   *aeruginosa* and *Nocardia* sp.), and a filamentous fungus was detected by histopathology. Similarly, three distinct

125   macroscopic patterns were observed according to the agent involved. In the first pattern (associated with *S. aureus*

126   and *P. aeruginosa*), nodular, yellowish, and firm structures (0.5-1.5 cm in diameter) were found in the middle of

127   the mammary parenchyma with purulent material at the center (Figure 3A). In the second pattern (associated with

128   *Nocardia* sp.), some quarters were firm, yellowish, and interspersed with dark red areas, with pronounced

129   mammary lobular pattern (Figure 3B). In the third pattern (associated with the fungus), multifocal to coalescing

130 nodules of 0.5 to 3.0 cm in diameter were observed, as well as markedly distended ducts filled with purulent

131 material (Figure 3C). Histologically, multiple pyogranulomas were observed in the mammary parenchyma

132 characterized by areas of necrosis, surrounded by a marked inflammatory infiltrate of intact and degenerate

133 neutrophils, epithelioid macrophages, multinucleated giant cells, lymphocytes and plasma cells, with peripheral

134 fibrosis (Figures 3D, 3E and 3F). In addition, in 14 of the 25 cases, a strongly eosinophilic, radiated material

135 (Splendore-hoeppli phenomen) was observed at the center of the pyogranulomas. In the middle of this material, in

136 11 of the 14 cases, large basophilic cocci were observed (*S. aureus*) (Figure 3D); in two cases, small basophilic

137 bacilli (*P. aeruginosa*); and in one case, septate and branched fungal structures, better visualized by the Grocott

138 technique (Figure 3F).

139 In both pyogranulomatous and necrosuppurative patterns associated with the isolation of *Nocardia* sp.

140 (8/10) or showing evidence of filamentous bacteria in lesions compatible with the agent (2/10), the bacteria were

141 better visualized by the ZN technique in 40% (4/10) of the cases and 80% (8/10) of the cases were positive in the

142 IHC (Figure 3E). In a case of pyogranulomatous mastitis, the H&E staining revealed bacillary bacteria that formed

143 small clusters, negative in IHC for *Nocardia* sp. and positive in IHC for *F. necrophorum*.

144 Grossly, abscedative mastitis was characterized by the formation of single or multiple abscesses in the

145 middle of the mammary parenchyma (Figure 4A). At histopathology, these were composed of areas of necrosis

146 with a large number of bacteria associated with a marked inflammatory infiltrate of intact and degenerate

147 neutrophils, as well as macrophages and lymphocytes, surrounded by a thick fibrous capsule (Figure 4B).

148 Squamous metaplasia of the glandular epithelium and multifocal areas of hemorrhage were frequently observed

149 adjacent to the abscess. *Trueperella pyogenes* was the predominant agent in this category and was isolated in pure

150 cultures (15/16).

151 On macroscopic examination, necrosuppurative lesions were characterized by a moderate evidence of the

152 mammary lobular pattern with yellowish areas in the middle of the parenchyma, occasionally filled by purulent

153 material, commonly associated with subcutaneous edema (Figure 4C). Histologically, there were multifocal areas

154 of coagulation necrosis in the mammary parenchyma associated with a marked inflammatory infiltrate of intact

155 and degenerate neutrophils, as well as fibrin deposition (Figure 4D), vascular fibrinoid necrosis, thrombosis, and

156 a large amount of bacteria. Hyperplasia and degeneration of epithelial cells and proliferation of fibrovascular tissue

157 were frequently observed. CNS and *E. coli* were the main etiological agents isolated in this category.

158 Granulomatous lesions presented a macroscopic pattern similar to that observed in the lymphoplasmacytic

159 mastitis, but two histological patterns were noted. The first pattern was characterized by an inflammatory infiltrate

160   composed of epithelioid macrophages, multinucleated giant cells, lymphocytes, and plasma cells distributed in a

161   multifocal to coalescent form and associated with discrete fibrosis (Figure 4E). In the second pattern, multiple

162   granulomas were observed in the middle of the mammary parenchyma, and were characterized by small areas of

163   necrosis, sometimes mineralized, surrounded by a moderate inflammatory infiltrate similar to that described in the

164   first pattern, and by a fibrous capsule (Figure 4F). There was no bacterial growth in this category. However, in

165   three quarters of the same cow, acid-fast bacilli could be identified through the ZN stain in the middle of the

166   mammary parenchyma and in the cytoplasm of multinucleated giant cells. These acid-fast bacilli were

167   morphologically compatible with *Mycobacterium* sp.

168   The main agents involved in cases of mastitis and their correlation to the type of lesion observed in the

169   mammary gland are described in Table 2.

170

171   **Discussion**

172   Mastitis is one of the primary diseases of dairy cows and is responsible for considerable economic losses

173   due to the decrease of the volume and quality of milk produced and the early culling of the cows (Bandeira et al.

174   2013; Acosta et al. 2016; Busanello et al. 2017). In this study, inflammatory lesions, involving at least one

175   mammary quarter, were detected in 133 out of 148 (89.9%) cows, and these lesions may have contributed to the

176   culling of these animals. Infection by bacterial agents, such as *S. aureus* and *Nocardia* spp., causes the destruction

177   of the secretory mammary epithelium with replacement by fibrous connective tissue, which leads to a decrease in

178   milk production and makes it impossible to maintain the cow in the productive cycle (Benites et al. 2002; Barkema

179   et al. 2006; Zhao and Lacasse 2008).

180   Bacteria are the main cause of bovine mastitis, acting through the ascending infection of the mammary

181   gland. However, the presentation forms of the disease can be influenced by factors related to the host, the

182   microorganism involved, and the environment (Benites et al. 2002; Schlafer and Foster 2016; Foster 2017). This

183   explains the great variety of the isolated agents and the morphological diagnoses observed during the histological

184   evaluation of the mammary glands conducted in this study.

185   The primary bacterial isolated from suppurative, mixed, and lymphoplasmacytic mastitis were

186   *Streptococcus* spp., CNS, *S. aureus*, *S. agalactiae*, *S. uberis* and *C. bovis*. The transmission of these bacteria is

187   associated with the habitat of these agents and occurs mainly from cow to cow during milking. *S. agalactiae* and

188   some strains of *S. aureus* are pathogens that must reside in the mammary gland and do not survive in the

189   environment. *S. uberis* can survive in both environments, however, it is mainly found in the feces and bedding

190   (Markey et al. 2013b; Foster 2017). CNS and *C. bovis* are commonly isolated from milk samples, and mainly

191   associated with cases of subclinical mastitis. CNS occurs as commensal in the skin of udder, and occasionally

192   cause opportunistic infections. However, some strains isolated from mastitis cases have invasive and toxin-

193   producing ability (Anaya-López et al. 2006; Markey et al. 2013b). *C. bovis* is considered a commensal of the

194   mammary gland, mainly in the teat canal, and can prevent infections by other agents (Markey et al. 2013b). This

195   may indicate that not all the isolations of these microorganisms in this research may be associated with the

196   inflammatory process identified.

197   Suppurative and mixed mastitis showed similar gross pattern, but they differed from lymphoplasmacytic

198   mastitis, mainly in relation to the aspect of mammary lobulation. Histologically, there was variation in the

199   inflammatory cell population involved among the patterns, as well as the intensity of the repair process (fibrosis),

200   which was scarce in the suppurative, mild in the mixed, and moderate in the lymphoplasmacytic mastitis.

201   Respectively, these pathological patterns indicate a probable acute, subacute, and chronic evolution of the lesions

202   and are in agreement with what is described by other authors (Benites et al. 2002; Schlafer and Foster 2016; Foster

203   2017). Hyperplasia and degeneration of epithelial cells have been frequently observed in these categories and are

204   mainly associated with the inflammatory process induced by *Streptococcus* spp. and *Staphylococcus* spp. (Schlafer

205   and Foster 2016; Foster 2017). Squamous metaplasia of the glandular epithelium, occasionally observed in this

206   study, is considered an evolution of the hyperplastic lesion and is related to the greater severity of the infectious

207   process (Foster 2017). Alveolar dilation, occasionally associated with the formation of fibrous polyps in the lumen

208   of the lactiferous ducts and sinuses, as well as in the gland cisternae, is also related to the chronicity of the

209   inflammatory process. The pathogenesis involves progressive periductal fibrosis that causes obstruction of milk

210   flow and consequent alveolar dilation (Benites et al. 2002; Schlafer and Foster 2016; Foster 2017).

211   Piogranulomatous mastitis was associated with different agents, as well as distinct lesions related to these

212   pathogens. *S. aureus* and *P. aeruginosa* produced a botryomycotic lesion pattern (Heyndrickx et al. 2012; Tessele

213   et al. 2014; Vinay et al. 2016), histologically characterized by the Splendore-Hoeppli phenomen. This reaction is

214   characterized by immunoglobulin aggregates which in cattle are observed primarily in chronic infections caused

215   by *S. aureus*, and in cases of actinobacillosis and actinomycosis (Tessele et al. 2014; Schlafer and Foster 2016).

216   Moreover, it can be observed in infections caused by *Nocardia* spp., *Mannheimia granulomatis*, agents associated

217   with mycetomas (as observed here in a case of fungal mastitis) and in some parasitic pyogranulomas (Tessele et

218   al. 2014).

219   Mastitis caused by *P. aeruginosa* in dairy cows is related to environmental contamination (Thompson et

220 al. 2001; Schlafer and Foster 2016). Botryomycosis associated with *P. aeruginosa* is commonly described in

221 humans (Heyndrickx et al. 2012; Vinay et al. 2016), but rarely reported in animals. In some known cases of

222 botryomycosis due to *P. aeruginosa* in cattle, infections were localized in the udder skin (Donovan and Gross

223 1984) and nasopharynx (Thompson et al. 2001). However, the presence of the bacteria in lesions with the

224 appearance of botryomycosis in the mammary parenchyma, as observed in two cases of this study, had never been

225 reported.

226   *Nocardia* is a microorganism often found in the soil, and it is transmitted through environmental

227 contamination or by the infusion of contaminated intramammary preparations (Pisoni et al. 2008; Schlafer and

228 Foster 2016). The infection usually occurs as outbreaks on farms with poor hygiene and handling conditions

229 (Pisoni et al. 2008). The agent mainly induces pyogranulomatous lesions (Pisoni et al. 2008; Schlafer and Foster,

230 2016), as observed in five cases described in this study. However, it can cause necrosuppurative lesions in acute

231 infections (Pisoni et al. 2008; Markey et al. 2013b), as observed in three cases.

232   Abscedative mastitis were characterized by single or multiple abscesses within the mammary

233 parenchyma, similar previous reports (Benites et al. 2002; Schlafer and Foster 2016). It was predominantly related

234 to the infection caused by *T. pyogenes*, a bacteria present in the skin and in the mucous membranes of several

235 animals (Markey et al. 2013b). Commonly, this type of mastitis was described in cows during the dry period and

236 in heifers, but currently it is also an important pathogen affecting lactating cows (Markey et al. 2013b; Ishiyama

237 et al. 2017). Often, cows with mastitis caused by *T. pyogenes* tend to be culled as these lesions are associated with

238 low rate of recovery of the mammary quarters, with extensive destruction of the parenchyma (Ishiyama et al.

239 2017). In agreement with this, we observed extensive lesions with a severe impairment of the mammary

240 parenchyma that justified the removal of the animals from the production system and sending them for slaughter.

241   The necrosuppurative pattern was characterized by acute lesions mainly associated with coliforms

242 (Hazlett et al. 1984; Markey et al. 2013b). *E. coli* and *Klebsiella* sp. produce endotoxins that cause tissue changes

243 through vascular damage, edema, hemorrhage, and thrombosis, similarly to those observed in this study. Such

244 agents may cause the death of the animal in some cases of environmental mastitis (Schiefer et al. 1976; Hazlett et

245 al. 1984; Schlafer and Foster 2016; Foster 2017).

246   No bacterial growth in the microbiological culture was observed in all cases of granulomatous mastitis.

247 Only in three of these cases, the association of macroscopic, histological, and histochemical findings allowed the

248 identification of mycobacteria, probably *Mycobacterium bovis*, since fast growing mycobacteria such as *M.*

249 *smegmatis* and *M. goodii* are not detected by lactoculture (Markey et al. 2013b). Mammary tuberculosis develops

250 slowly with progressive enlargement of the gland which becomes firm. However, most of the time, there is no

251 formation of classic miliary lesions as in other organs (Schlafer and Foster 2016), and this is consistent with the

252 findings of this study.

253   The high number of mammary quarters with granulomatous lesion without identifiable etiology is similar

254 to idiopathic granulomatous mastitis described in humans, that the pathogenesis is not fully elucidated. Hypotheses

255 put forward to explain the etiology of idiopathic granulomatous mastitis include trauma, similar to that observed

256 in the cases of testicular sperm granulomas and granulomatous thyroiditis, as well as hormonal imbalances.

257 Hyperprolactinemia and imbalance in the estrogen-progesterone ratio may lead to increase in protein secretion

258 causing ectasia and rupture of the alveoli and ducts with extravasation of this secretion and consequent

259 development of granulomatous inflammation (Altintoprak et al. 2014).

260   The results allow us to conclude that mixed, lymphoplasmacytic, and suppurative mastitis were the main

261 histopathological patterns observed with involvement of *Streptococcus* spp., CNS, *S. aureus*, *S. agalactiae*, *S.*

262 *uberis* and *C. bovis*. The pyogranulomatous pattern presented different forms depending on the agent involved,

263 and was primarily associated with *S. aureus* and *Nocardia* sp. The cases of abscedative mastitis were characterized

264 by extensive destruction of the mammary parenchyma predominantly caused by *T. pyogenes*. The

265 necrosuppurative pattern was characterized by acute lesions predominantly associated with environmental bacteria

266 producing endotoxins, such as *E. coli*. Granulomatous mastitis had the lowest frequency of cases and was

267 occasionally associated with *Mycobacterium* sp.

268

272

273 **Statement of animal rights**

274 The manuscript does not contain clinical studies or patient data.

275

276 **Conflicts of interest**

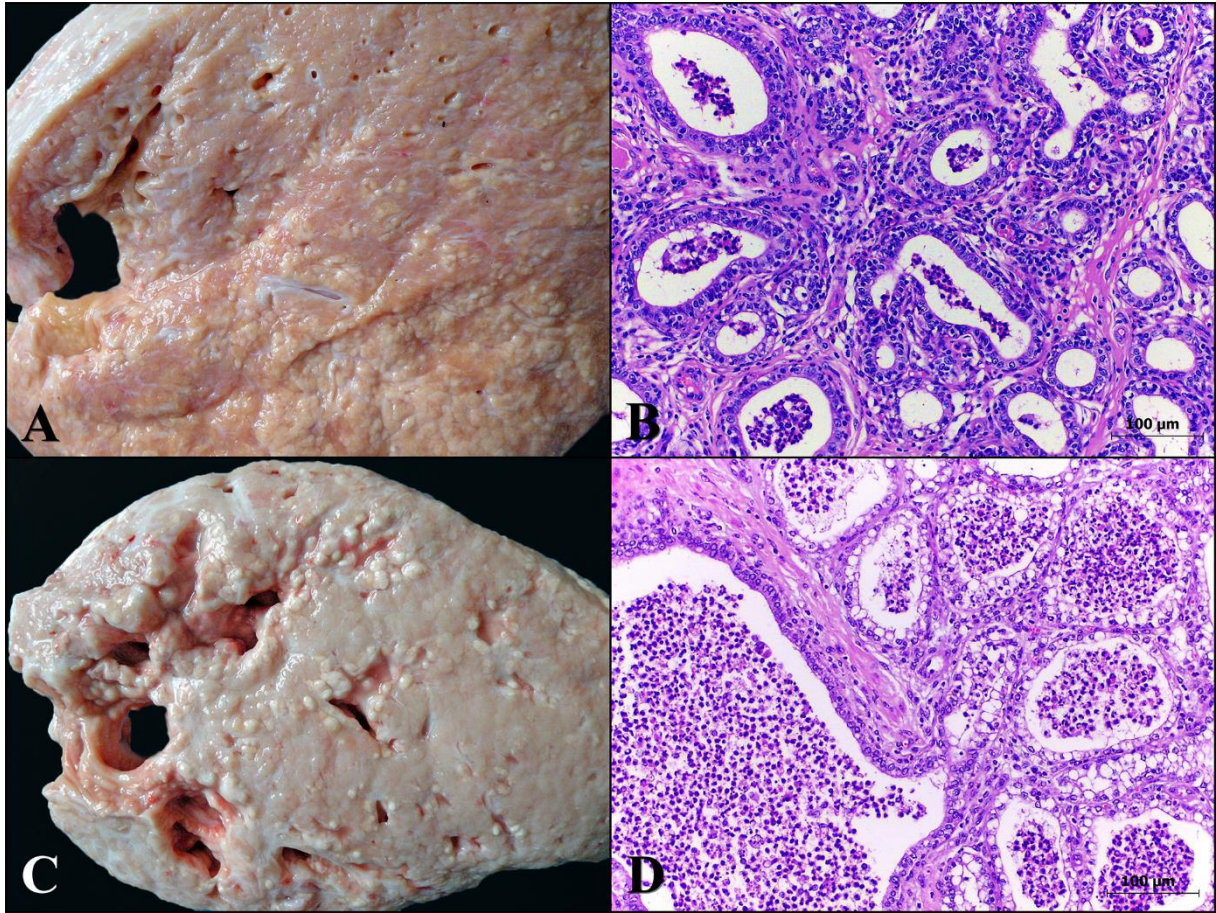277 The authors declare no conflicts of interest.

278

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Fig. 1. Patterns of mastitis in dairy cows. **a** Mixed mastitis. There is evidence of the mammary lobular pattern with the presence of yellowish nodules in the middle of the parenchyma, ranging from 0.2 to 0.5 cm in diameter, and interspersed by thin white septa. **b** Mixed mastitis. Image is showing a moderate inflammatory infiltrate composed of neutrophils within the alveoli, in addition to multifocal infiltration of neutrophils, lymphocytes, plasma cells, and macrophages in the interstitium, interspersed by discrete fibrosis. Hematoxylin and eosin (H&E). x20. **c** Suppurative mastitis. Gross pattern similar to that described in A, with nodules protruding into the lumen of the lactiferous ducts and the cisternae of the mammary gland. **d** Suppurative mastitis. There is a marked infiltration of intact and degenerate neutrophils within the alveoli and ducts, associated with pronounced vacuolization of epithelial cells (degeneration). H&E. x20.
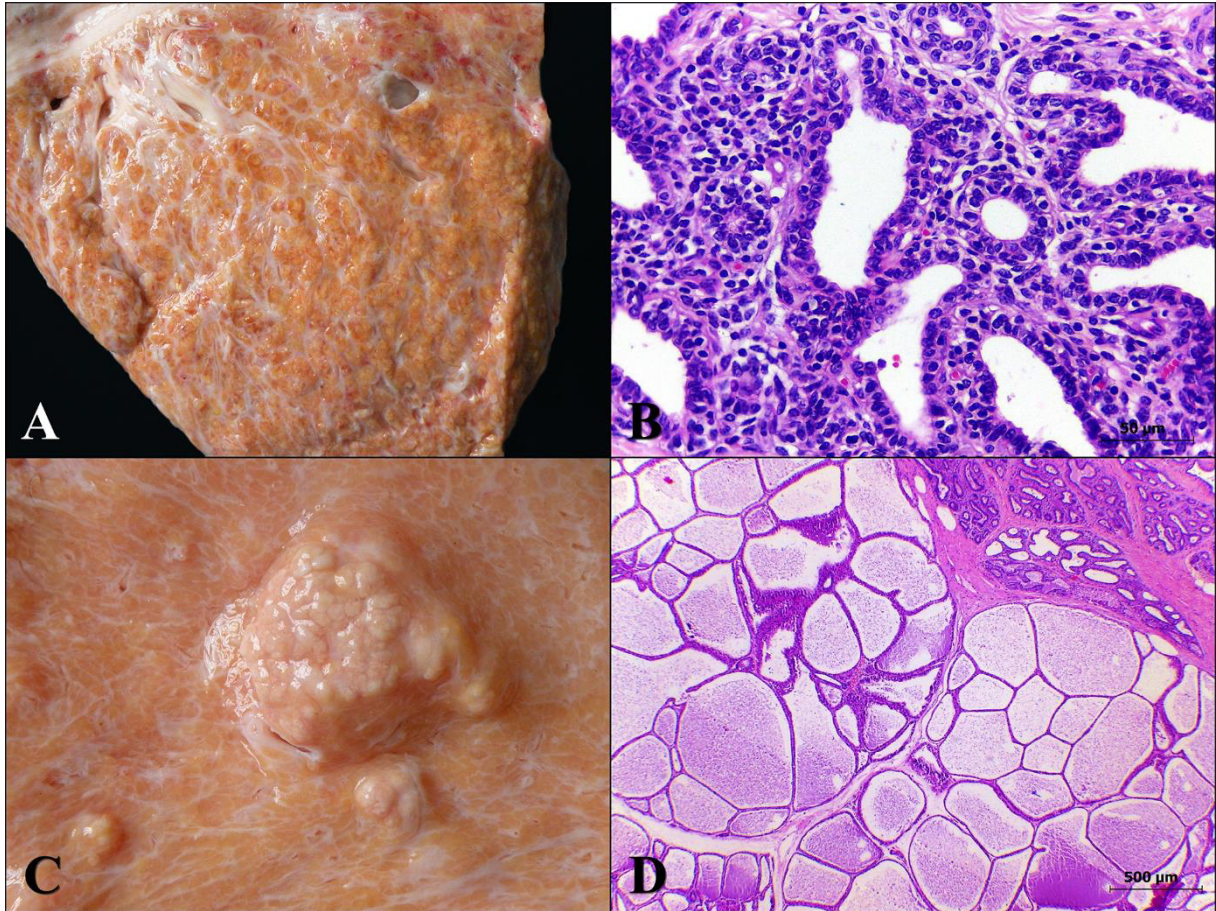
Fig. 2. Lymphoplasmacytic mastitis in dairy cows. **a** Mammary quarter with diminished lobulations and thick white septa that dissect the parenchyma. **b** There is moderate, multifocal and interstitial inflammatory infiltrate composed of lymphocytes and plasma cells associated with moderate fibrosis. H&E. x40. **c** Nodular, white and polypoid-like formations are seen in the middle of the mammary parenchyma. **d** Several markedly dilated alveoli, sometimes covered by hyperplastic epithelium, are noted. H&E. x4.

362
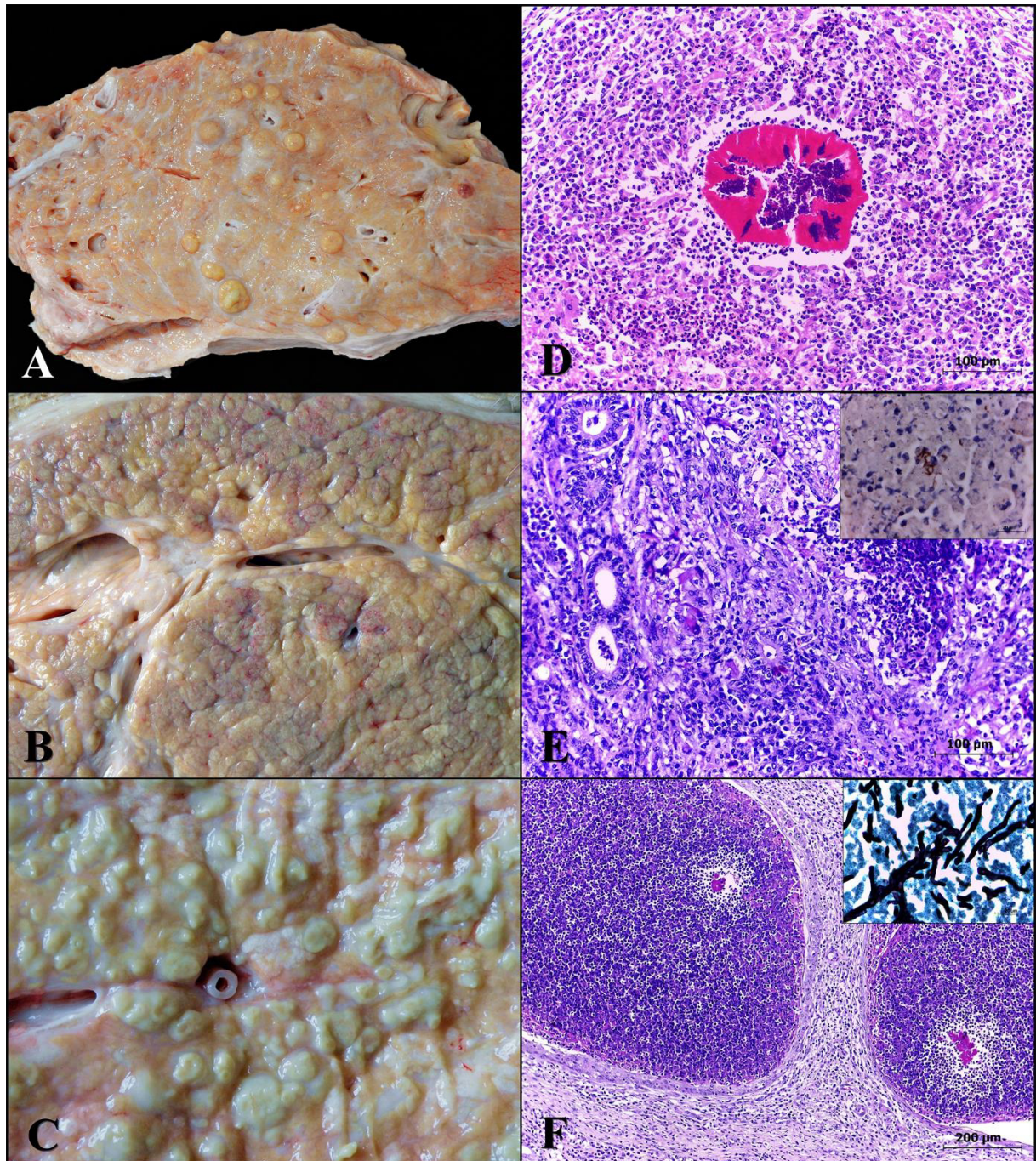
363 **Fig. 3.** Pyogranulomatous mastitis in dairy cows. **a** Nodular, yellowish, and firm structures are found in the middle

364 of the mammary parenchyma, ranging from 0.5 to 1.5 cm in diameter, with purulent material at the center (lesion

365 associated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*). **b** There is a firm and yellowish mammary

366 quarter interspersed with dark red areas with evidence of the lobular pattern (lesion associated with *Nocardia* sp.).

367 **c** Multifocal to coalescing nodules, of varying sizes, filled with purulent contents (fungal mastitis) are observed in

368 the middle of the mammary parenchyma. **d** A marked inflammatory infiltrate of intact and degenerate neutrophils,

369 epithelioid macrophages, multinucleated giant cells, lymphocytes, and plasma cells is seen in the mammary

370 parenchyma. There is also a strongly eosinophilic, radiated material (Splendore-hoeppli phenomen), which

371   contains large basophilic cocci in the center (*S. aureus*). H&E. x20. **e** A focal area of necrosis associated with a

372   marked inflammatory infiltrate of intact and degenerate neutrophils, macrophages, lymphocytes, plasma cells, and

373   occasional multinucleated giant cells is observed in the middle of mammary parenchyma. H&E. x20. Inset,

374   positive immunolabeling for *Nocardia* sp. Immunohistochemistry. x100. **f** There are marked dilated alveoli, filled

375   by intact and degenerate neutrophils, as well as necrotic debris, associated with Splendore-hoeppli phenomen.

376   Marked fibrosis interspersed by a moderate inflammatory infiltrate of macrophages, lymphocytes, plasma cells

377   and occasional multinucleated giant cells is seen in the interstitium. H&E. x10. Inset, there are septate and branched

378   fungal structures, strongly impregnated by silver. Grocott Methenamine Silver. x100.
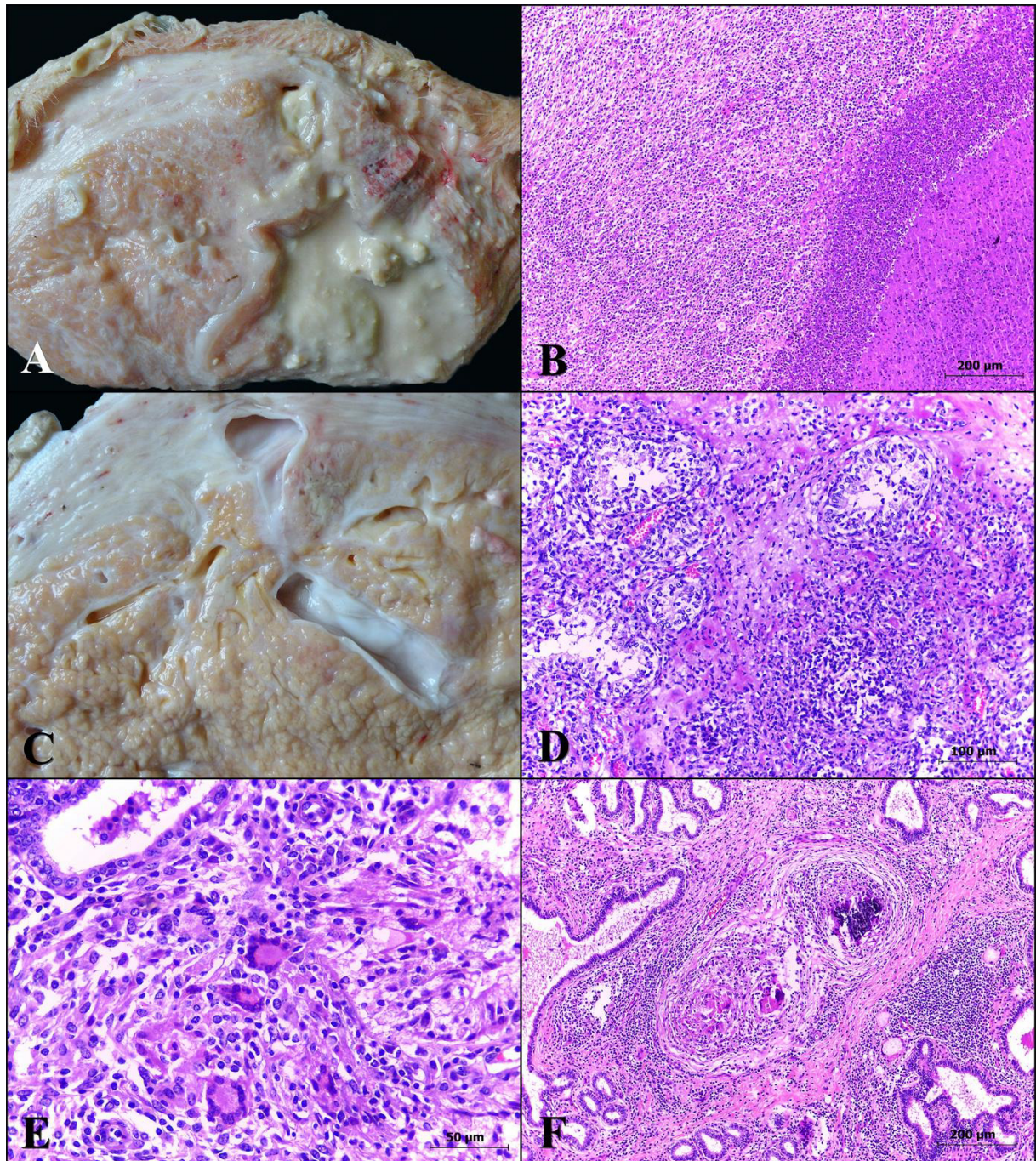
Fig. 4. Patterns of mastitis in dairy cows. **a** Abscedative mastitis. In the middle of the parenchyma, cavitations filled with purulent content and surrounded by a fibrous capsule (abscesses) are observed. **b** Abscedative mastitis. The layers of an abscess are observed: a necrotic area on the right is surrounded by a marked amount of intact and degenerate neutrophils and more externally (to the left), marked fibrosis interspersed by macrophages, lymphocytes, and plasma cells. H&E. x10. **c** Necrosuppurative mastitis. There is moderate evidence of the mammary lobular pattern, with a yellowish area in the middle of the parenchyma. **d** Necrosuppurative mastitis. Multifocal areas of necrosis of the mammary parenchyma are observed, associated with a marked inflammatory infiltrate of intact and degenerate neutrophils with an abundant deposition of fibrin. H&E. x20. **e** Granulomatous

388 mastitis. In the mammary parenchyma, there is a marked inflammatory infiltrate composed of epithelioid

389 macrophages, multinucleated giant cells, lymphocytes and plasma cells. H&E. x40. **f** Granulomatous mastitis.

390 Multifocal granulomas are found in the middle of the mammary parenchyma, characterized by areas of mineralized

391 necrosis surrounded by an infiltrate, similar to that described in e, with moderate peripheral fibrosis. H&E. x10.

392   Table 1: Morphological aspects of bovine mammary lesions and pathogenic agents identified.

Agent	N. of quarters	Agent	N. of quarters
Suppurative mastitis		**Lymphoplasmacytic mastitis**	
Streptococcus spp.	18	*Streptococcus* spp.	21
Coagulase-negative *Staphylococcus*	9	Coagulase-negative *Staphylococcus*	21
Staphylococcus aureus	3	*Corynebacterium bovis*	21
Outros estreptococos (S. *agalactiae*,	5	*Staphylococcus aureus*	8
S. *uberis* e *S. dysgalactiae*)		*Streptococcus uberis*	8
Others (*C. bovis*, *E. coli*, *Klebsiella* sp.,	13	*S. agalactiae*, *Proteus* sp.,	5
T. pyogenes, *Proteus* sp., and *Bacillus* sp.)		*Escherichia coli*, and *Bacillus* sp.	
Necrosuppurative mastitis		**Mixed mastitis**	
Coagulase-negative *Staphylococcus*	6	*Streptococcus* spp.	36
Escherichia coli	4	*Staphylococcus aureus*	22
Gram negative rod-shaped	4	Coagulase-negative *Staphylococcus*	22
Nocardia sp.	3	*Corynebacterium bovis*	18
Klebsiella sp.	2	*Streptococcus agalactiae*	8
Bacillus sp.	2	*S. uberis* and *S. dysgalactiae*	5
Streptococcus spp., *S. agalactiae*,	6	Others (Gram negative rod-shaped, *Bacillus* sp.,	16
Proteus sp., and *C. bovis*.		*Nocardia* sp, *T. pyogenes*, and *E. coli*)	
Abscedative mastitis		**Pyogranulomatous mastitis**	
Trueperella pyogenes	16	*Staphylococcus aureus*	11
Staphylococcus aureus	2	*Nocardia* sp.	5
Streptococcus spp., and *S. dysgalactiae*	4	*Pseudomonas aeruginosa*	2
Corynebacterium bovis	1	*Fusobacterium necrophorum*	1
		Filamentous fungus	1

393   * Bacterial agents isolated from mammary quarters without histopathological lesions were disregarded.

394   Table 2: Bacterial agents more frequently identified in bovine mastitis and the patterns of associated lesions.

Bacteria	Frequency	Associated lesions in descending order
Streptococcus spp.	18.3% (79/432)	Mixed, lymphoplasmacytic and suppurative mastitis
Coagulase-negative *Staphylococcus*	13.4% (58/432)	Lymphoplasmacytic, mixed and suppurative mastitis
Staphylococcus aureus	10.6% (46/432)	Mixed, pyogranulomatous and lymphoplasmacytic mastitis
Corynebacterium bovis	10.2% (44/432)	Lymphoplasmacytic and mixed mastitis
Trueperella pyogenes	4.9% (21/432)	Abscedative mastitis
Streptococcus uberis	3.0% (13/432)	Lymphoplasmacytic mastitis
Streptococcus agalactiae	2.8% (12/432)	Mixed mastitis
Nocardia sp.	2.5% (11/432)	Pyogranulomatous and necrosuppurative mastitis
Escherichia coli	1.6% (7/432)	Necrosuppurative mastitis

395   * Bacterial agents isolated from mammary quarters without histopathological lesions were disregarded.

3. ARTIGO 2

Nesse item é apresentado o artigo intitulado:

Molecular and pathological characterization of teat papillomatosis in dairy cows in Southern Brazil

Ronaldo M. Bianchi, Christian D. B. T. Alves, Claiton I. Schwertz, Welden Panziera, Cíntia De Lorenzo, Fernando S. da Silva, Bianca S. de Cecco, Cláudio W. Canal, Saulo P. Pavarini e David Driemeier

(Artigo será submetido ao periódico *Brazilian Journal of Microbiology*)

1 **Molecular and pathological characterization of teat papillomatosis in dairy cows in Southern**

2 **Brazil**

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13 Running title: Teat papillomatosis in dairy cattle

1   **Abstract.**

2   Teat papillomatosis is caused by different bovine papillomavirus (BPV) types and is especially

3   important for dairy cows, because it results in severe damage to the health and structure of the

4   mammary gland. This work describes the molecular and pathological aspects of teat papillomatosis in

5   dairy cows in Southern Brazil. Samples of teat papillomas were collect of 73 slaughtered dairy cows.

6   Fragments of the lesions were collected in individual pools per animal and subjected to molecular

7   analysis. Teats with the remaining lesions were fixed in 10% neutral buffered formalin, routinely

8   processed for histopathology and stained with hematoxylin and eosin (H&E). Papillomatous lesions

9   were characterized by three macroscopic patterns: exophytic (5 [6.9%]), flat (29 [39.7%]) and mixed

10   (39 [53.4%]). Histologically, all samples were identified as squamous papillomas. Based on the

11   molecular analysis, eight classical BPV types (BPVs 4, 6, 7, 8, 9, 10, 11 and 12) were identified in 27

12   samples, six previously reported putative BPV types in 17 samples, and 10 putative new BPV types in

13   15 samples. Four sequences did not allow the classification and 10 were negative. There was no

14   relation between the gross pattern and the BPV type identified, and all samples were characterized by

15   squamous papillomas at the histological examination. However, different BPV types were identified

16   and demonstrated a great diversity of BPVs associated with teat papillomatosis in dairy cows in

17   Southern Brazil.

18   **Keywords:** dairy cattle; papillomatosis; BPV; viral diseases; veterinary pathology; PCR.

1   **Introduction**

2   Papillomavirus (PVs) are non-enveloped, double-stranded DNA viruses, belonging to the

3   Papillomaviridae family [11, 22, 24]. In cattle, they cause benign cutaneous papillomatous lesions,

4   which may involve the teats. In addition, they are also associated with malignant tumors in the bladder

5   and upper digestive tract [7, 15, 17].

6   Teat papillomatosis is commonly described in dairy cattle and this may result in damage to the

7   health and structure of the mammary gland. Papillomas may be large enough to cause interference in

8   the milking process and milk flow, especially when they are located near the sphincter of the teat,

9   predisposing to the occurrence of mastitis. In addition, ulceration and rupture of the lesions may cause

10   bleeding and distortion of the lactiferous ducts [5, 6, 8, 14, 18, 24].

11   Currently, according to the papillomavirus genome database (PaVE) [21], there are 24 fully

12   characterized bovine papillomavirus (BPV) types which are classified into five genera.

13   *Deltapapillomavirus* genus, with one species (*Deltapapillomavirus 4*) and four types (BPVs 1, 2, 13

14   and 14); *Epsilonpapillomavirus* genus with one species (*Epsilonpapillomavirus 1*) and two types

15   (BPVs 5 and 8); *Dyoxipapillomavirus* genus with one species (*Dyoxipapillomavirus 1*) and one type

16   (BPV7); *Dyokappapilomavirus* genus with three types (BPVs 16, 18 and 22); and *Xipapillomavirus*

17   genus, composed by the species *Xipapillomavirus 1*, which encompasses BPVs 3, 4, 6, 9, 10, 11 and

18   15, and *Xipapillomavirus 2*, which encompasses BPV12. The BPVs 17, 20, 23 and 24 also belong to

19   the *Xipapillomavirus* genus but they do not present species demarcation, and there is also a new

20   unclassified genus that includes the BPVs 19 and 21.

21   Although there are 24 BPV types identified, most of these recently characterized, this number

22   still contrasts with the more than 200 human papillomavirus (HPV) types described [21]. Therefore,

23   this work aims to describe the molecular and pathological aspects of teat papillomatosis in dairy cows

24   in Southern Brazil, in addition to reporting the identification of 10 putative new BPV types.

1 **Materials and Methods**

2 **Sampling and histopathology**

3   From August of 2016 to March of 2017, the slaughter of dairy cows was carried out in two

4 slaughterhouses located in the state of Rio Grande do Sul, Southern Brazil. The mammary glands were

5 inspected and 73 cows with papillomatous lesions in the teats and, occasionally, also in the udder were

6 selected. In sequence, the teats, along with a fragment of the skin of the udder base, were collected.

7 Small fragments of the papillomas of each cow were collected in pools, composing one sample per

8 cow. These were frozen at -20°C and subjected to molecular analysis. The remaining material was

9 fixed in 10% neutral buffered formalin, routinely processed for histopathology and stained with

10 hematoxylin and eosin (H&E).

11 **DNA isolation**

12   Papilloma specimens were ground with sterile sand in 10 mL of phosphate buffered saline

13 (PBS) (pH 7.4), centrifuged at 720 x g for 10 min and 1000 µL of the supernatant was stored at -20 °C

14 for molecular analysis. DNA was isolated of 100 µL using a phenol-chloroform following usual

15 procedures [25] and eluted in 50 µL of ultrapure water. The quality and quantity of the DNA were

16 assessed through spectrophotometry and fluorometry performed with NanoDrop™ (Thermo Fisher

17 Scientific) and Qubit™ (Thermo Fisher Scientific) respectively.

18 **PCR and Sanger sequencing**

19   Partial amplification of the L1 gene was performed with the forward oligonucleotide FAP59

20 (5'-TAA CWG TIG GIC AYC CWT ATT-3') (Position in BPV strain X02346: 5712-5752) and the

21 reverse oligonucleotide FAP64 (5'-CCW ATA TCW VHC ATI TCI CCA TC-3') (Position in BPV

22 strain X02346: 6206-6185) [13]. Briefly, 100 ng of extracted DNA was mixed with [1x] PCR buffer,

23 20 pmol of each primer, 2 mM of $MgCl_2$, 200 µM of dNTPs, 1 U of GoTaq® DNA Polymerase

24 (Promega, Madison, WI, USA) in a total volume of 25 µL adjusted with ultrapure water. After an

25 initial incubation at 95°C for 5 min, 40 cycles were carried out consisting of denaturation at 95°C for 1

26 min, annealing at 50°C for 1 min, and extension at 75°C for 1min. The PCR products were purified

27 using PureLink™ Quick PCR Purification Kit (Invitrogen, Carslbad, CA, USA). Aliquots from the

1 reactions were analyzed by electrophoresis in 1 % agarose gels stained with GelRed Loading Buffer

2 (Biotium Inc., Hayward, CA, USA), and examined under UV light. Both strands were sequenced to

3 confirm the PCR results with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster

4 City, CA, USA) using a BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster

5 City, CA, USA).

6 **Sequence analysis**

7 The sequences were compared with all sequences in the GenBank through Basic Local

8 Alignment Search Tool (BLAST) to determine the sequence identity [26]. Representative sequences of

9 the ruminants PV sequences were retrieved from GenBank. Nucleotide alignments were performed

10 using MUSCLE software [12]. The percentage similarity of the PV sequences determined in this work

11 in comparison with previously determined PV sequences was estimated using MEGA6 computer

12 software (version 6) [23]. With the intend to verify if the putative new BPV types sequences are the

13 same BPV, was constructed a matrix using GENEIOUS software (version 9) that displayed the

14 percentage of identity among the sequences.

15 The taxonomy criteria of the BPV samples was conducted based on the L1 gene [4]. The

16 entire L1 gene sequence must be different more than 10% of nucleotide pairwise identity of the closest

17 know type to be considered a new type. The putative new PV types were defined if the nucleotide

18 identity displayed less than 90% similarity with the L1 gene fragment of all PV types already

19 classified [11, 20].

20 **Results**

21 **Gross and histopathological findings**

22 In the great majority of the 73 cows, the four teats were affected by papillomatous lesions

23 (Fig. 1a). Grossly, the papillomas were characterized by three patterns. The first one (pattern 1) was

24 identified in 5 cows (6.9%), and consisted of brown to blackish, exophytic projections, with varying

25 sizes and vegetative, digitiform or filiform aspect (Fig. 1b). The second pattern (pattern 2) was

26 observed in 29 cows (39.7%) and was characterized by lightly elevated, whitish projections, with a flat

27 to round surface (Fig. 1c). The third and most frequent pattern (pattern 3 or mixed) was observed in 39

1   cows (53.4%). This was characterized by a mixed presentation. In the same teat or the same cow,

2   papillomatous lesions similar to those described in the patterns 1 and 2 were observed (Fig. 1d).

3   Histologically, patterns 1 and 2 showed very similar morphological characteristics, differing

4   only in surface appearance, which was vegetating to digitiform in pattern 1(Fig. 2a) and flat to

5   undulating in pattern 2 (Fig. 2b). All lesions were identified as squamous papillomas and were

6   characterized by marked epidermal hyperplasia, ortho or parakeratotic hyperkeratosis and increased of

7   keratohyaline granules. Swollen keratinocytes, with a lightly eosinophilic cytoplasm and a pyknotic

8   nucleus, surrounded by a clear halo (koilocytes) were observed in the spinous and granular layers of

9   epidermis in 28 of 73 cows (Fig. 2c). In addition, intranuclear amphophilic inclusion bodies in

10 keratinocytes were only observed in one cow (Fig. 2d).

11 **PCR, Sanger sequencing**

12 The 63 of the 73 papilloma samples generated a 480 bp-fragment from the L1 gene by

13 conventional PCR using oligonucleotide pairs FAP59/FAP64 [13]. All the fragments were submitted

14 for Sanger sequencing to confirm the PCR results and to analyze the nucleotide similarity of the L1

15 gene fragment. The sequences were discriminated in BPV type, previously reported putative BPV type

16 and putative new BPV types after comparison of the homology with previously published PV types,

17 putative PV types and between putatives new BPV types detected in this study. Those sequences of

18 which their degree of identity was not greater than 90% with classical BPV types were divided into

19 previously reported putative BPV types and putative new BPV types. The previously reported putative

20 BPV types were those that did not fit the previous measure but have a degree of identity above 90%

21 with other BPV types already reported. Finally, sequences classified as putative new BPV types were

22 those whose degree of nucleotide similarity was not greater than 90%, with no sequence retrieved

23 from the genetic database.

24 The BPV types were detected in 27 sequences and between them the most frequent was BPV8

25 (12/27), follow by BPVs 6 and 7, both with four detections (Table 1). The previously reported putative

26 BPV types represented 17 sequences and the most frequent was the BAPV8 (6/17) (Table 2). The

27 putative new BPV types were found in 15 sequences, ranging 74.3% to 89.1% of nucleotide similarity

1   within PVs available in the GenBank database (Table 3). As shown in the matrix of identity (Fig. 3)

2   there are 10 putative new BPV types, since the sequences AP3878-16, AP3881-16, AP4169-16,

3   AP4829-16 and AP781-17 display more than 90% of nucleotide similarity to each other. The same

4   occur with the sequences AP4144-16 and AP4174-16. There were no homologies with PVs from other

5   hosts between the sequences of this study, and four sequences did not allow the classification, because

6   the fragments were too short (above 100 nt).

7   **Discussion**

8   The diagnosis of teat papillomatosis in dairy cows was based on the gross and microscopic

9   findings and the involved BPV types determined through molecular analysis, which allowed the

10   identification of a wide variety of BPVs.

11   The involvement of the four teats was observed in most cows. This is a frequent feature of teat

12   papillomatosis described in dairy cows [18] and may be associated with the infection process. To

13   infect an animal, BPVs require micro abrasions or cutaneous wounds. In addition, they are highly

14   contagious and may be a herd problem, since their transmission occurs easily from one bovine to

15   another by direct contact, or indirectly through fomites, insects and pastures [6, 18, 19]. For dairy

16   cattle, the milking process also plays an important role in the transmission through the equipment and

17   hands of milkers [14].

18   Different patterns of teat papillomatosis were observed in the macroscopic evaluation.

19   However, the histological changes were similar among the patterns and were characterized by

20   squamous papillomas, because only the epidermis was affected. In general, the BPVs of the genus

21   *Deltapapillomavirus* (1, 2, 13 and 14) induce the formation of fibropapillomas since they infect the

22   epidermis and the dermis. These four types were not observed in this study, in the same way as BPV5,

23   which is capable of causing both fibropapillomas and squamous papillomas [11, 15, 16, 17]. BPV8 is

24   also able to induce both types of papillomas because it belongs to the same genus of BPV5

25   (*Epsilonpapillomavirus*) [19]. However, despite being identified in 12 cows, all lesions associated

26   with BPV8 were squamous papillomas.

1   In the present study, BPV DNA was amplified and sequenced in 59 of 73 teat papillomas

2   samples of dairy cows, allowing the identification of 8 classical BPV types (n=27), 6 previously

3   reported putative BPV types (n=17) and 10 putative new BPP types (n=15). The identification of

4   different BPV types reflects the wide genetic diversity of the virus related to teat papillomatosis in

5   dairy cows in the analyzed region. However, despite the wide variability of BPVs identified in this

6   study, the same histological changes of papillomas were observed in all samples [19] and there was no

7   relation between the gross pattern and the BPV type identified. The observation of koilocytes in the

8   histological analysis, although not considered pathognomonic of the viral infection, is a probable

9   indicative of the cytopathic effect of the papillomavirus in the tissues [2, 5].

10   PCR assays using degenerate primers (FAP59 / FAP64) for the amplification of partial

11   fragments of the BPV L1 gene, followed by amplification of the product, has been widely used to

12   determine the presence of different BPV types in cattle herds of different geographical regions [1, 10,

13   20, 22, 24]. When compared to the use of specific primers, the PCR with degenerate primers presents

14   a lower level of sensitivity [22], which may justify the 10 negative samples observed in this study.

15   However, it is an important tool for the identification of putative new BPV types [17, 22].

16   Eight classical BPV types (BPVs 4, 6, 7, 8, 9, 10, 11 and 12) were identified in this study.

17   Among these, BPVs 6, 7, 9 and 10 are widely correlated to papillomas in the teats or in the udder of

18   cattle [16,18,19,20,24]. However, BPV8 is rarely associated with teat papillomatosis [20] but with

19   papillomas in other regions of the skin [3, 10, 19]. In this work, BPV8 was identified in 12 cows

20   which demonstrates a wide capacity of infection in different anatomical sites.

21   The wide diversity of BPV types identified in our study resembled other researches conducted

22   in different regions of Brazil focusing on cutaneous and teat papillomatosis of cattle [2, 3, 9, 10, 22,

23   24]. Our results reinforce the importance of genotyping studies for the identification of classical BPVs,

24   as well as, of new BPV types, since the immune response against the papillomavirus is specific type

25   [24] and fundamental for the control of the disease.

26   Teat papillomatous lesions were characterized by three gross patterns (exophytic, flat and

27   mixed), and the mixed pattern was identified in more than 50% of the cows. Histologically, all lesions

were characterized by squamous papillomas. Eight classical BPV types, 6 previously reported putative BPV types and 10 putative new BPP types were identified in the molecular analysis, which indicates a great variability of viral types and emphasizes the importance of teat papillomatosis in dairy cows.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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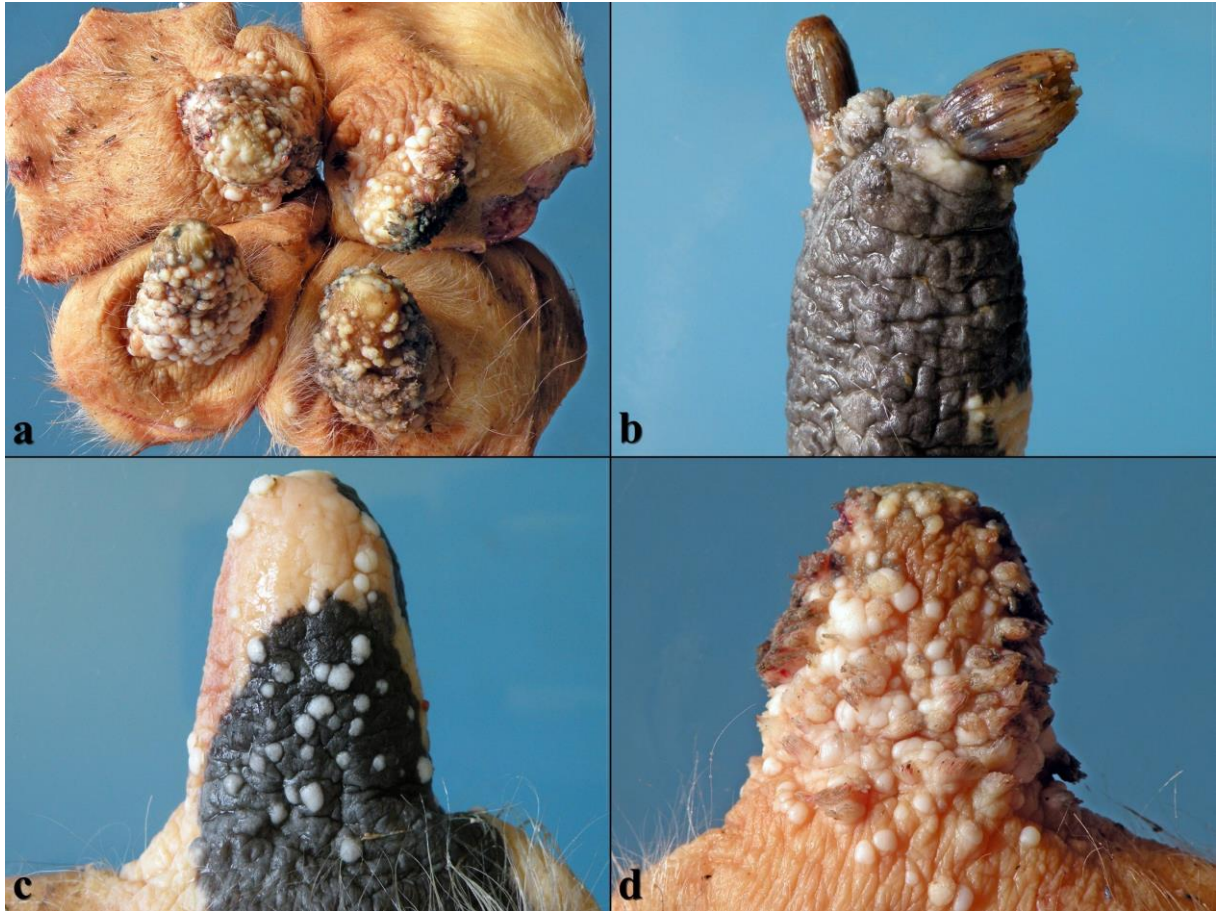
Fig. 1. Gross findings of teat papillomatosis in dairy cows. **a** All four teats are affected by papillomas. **b** Brown to blackish and exophytic projections, with vegetative aspect are observed in the surface of the teat (pattern 1). **c** Flat, lightly elevated and whitish projections are observed in the surface of the teat (pattern 2). **d** Papillomatous lesions similar to those described in b and c are observed in the same teat (pattern 3).
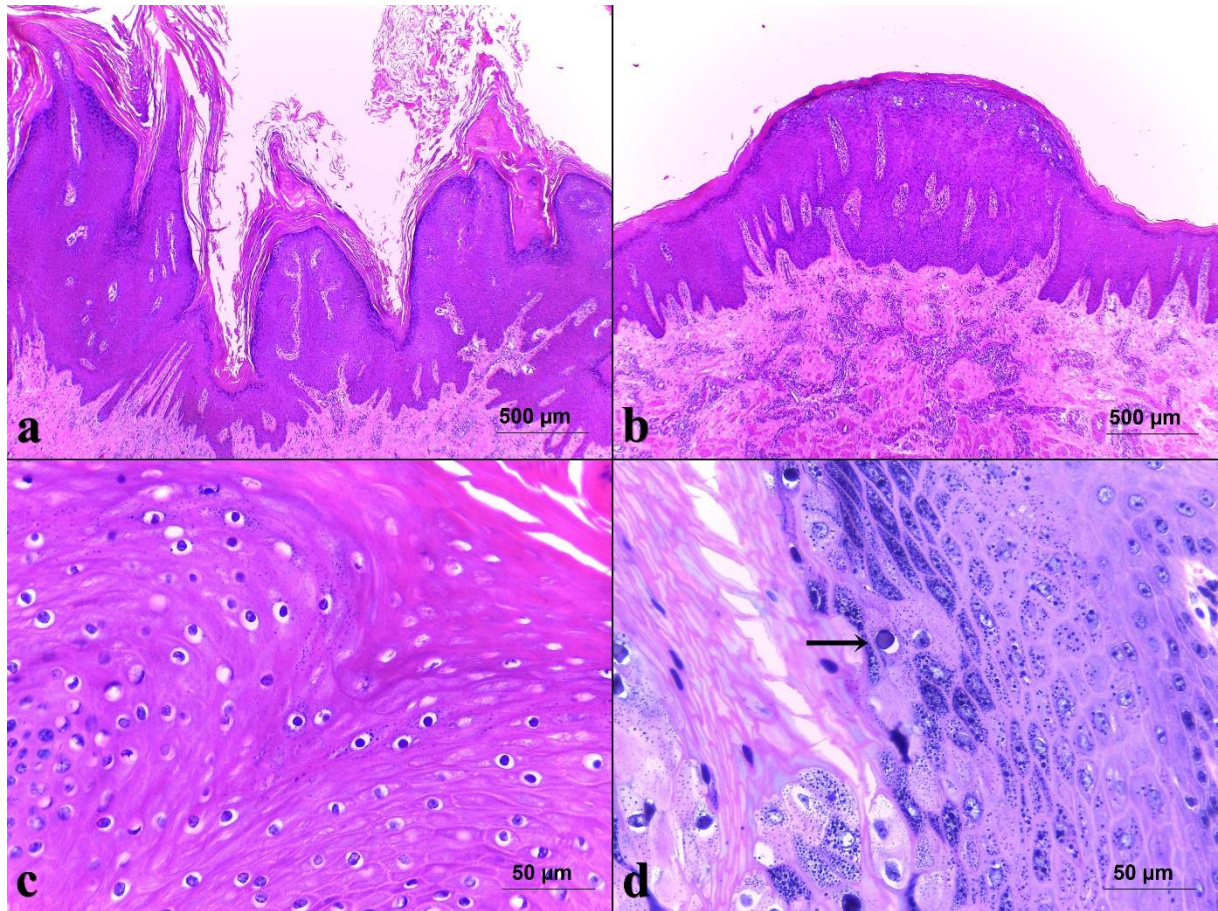
Fig. 2. Histological findings of teat papillomatosis in dairy cows. **a** Histological aspect of the gross pattern 1 characterized by marked epidermal hyperplasia and orthokeratotic hyperkeratosis with vegetating to digitiform surface. Hematoxylin and eosin (H&E), 4x. **b** Histological aspect of the gross pattern 2 characterized by marked epidermal hyperplasia and orthokeratotic hyperkeratosis with flat surface. H&E, 4x. **c** There are many swollen keratinocytes, with a lightly eosinophilic cytoplasm and a pyknotic nucleus, surrounded by a clear halo (koilocytes) in the spinous and granular layers of the epidermis. H&E, 40x. **d** In the center of the figure, there is a keratinocyte with an intranuclear amphophilic inclusion body (arrow). Note that there is also marked increase of keratohyaline granules. H&E, 40x.

	AP771-17	AP4157-16	AP4167-16	AP3892-16	AP3899-16	AP3885-16	AP4835-16	AP781-17	AP4169-16	AP3878-16	AP3881-16	AP4829-16	AP4178-16	AP4144-16	AP4174-16
AP771-17		66.0%	29.7%	33.7%	30.8%	35.0%	31.6%	36.5%	35.1%	32.3%	36.1%	36.2%	32.5%	31.2%	32.2%
AP4157-16	66.0%		28.4%	29.7%	29.0%	32.5%	31.3%	36.1%	33.4%	32.6%	33.7%	33.8%	29.9%	29.3%	29.7%
AP4167-16	29.7%	28.4%		56.0%	61.8%	68.1%	45.3%	52.5%	51.7%	50.8%	51.8%	51.9%	52.5%	58.5%	56.2%
AP3892-16	33.7%	29.7%	56.0%		57.0%	65.7%	54.5%	63.3%	65.1%	63.5%	65.8%	65.6%	68.4%	64.3%	63.0%
AP3899-16	30.8%	29.0%	61.8%	57.0%		72.1%	56.7%	66.3%	66.0%	65.3%	65.5%	66.0%	64.0%	64.5%	65.7%
AP3885-16	35.0%	32.5%	68.1%	65.7%	72.1%		54.2%	64.3%	65.4%	64.4%	66.7%	66.9%	67.3%	67.7%	69.0%
AP4835-16	31.6%	31.3%	45.3%	54.5%	56.7%	54.2%		79.5%	72.4%	77.4%	73.3%	73.1%	62.4%	61.9%	60.0%
AP781-17	36.5%	36.1%	52.5%	63.3%	66.3%	64.3%	79.5%		97.6%	96.4%	97.1%	97.6%	77.3%	69.9%	69.9%
AP4169-16	35.1%	33.4%	51.7%	65.1%	66.0%	65.4%	72.4%	97.6%		98.3%	96.0%	96.1%	75.1%	70.2%	70.7%
AP3878-16	32.3%	32.6%	50.8%	63.5%	65.3%	64.4%	77.4%	96.4%	98.3%		98.6%	98.3%	76.6%	69.3%	70.6%
AP3881-16	36.1%	33.7%	51.8%	65.8%	65.5%	66.7%	73.3%	97.1%	96.0%	98.6%		99.6%	78.3%	70.2%	71.9%
AP4829-16	36.2%	33.8%	51.9%	65.6%	66.0%	66.9%	73.1%	97.6%	96.1%	98.3%	99.6%		78.4%	70.2%	71.8%
AP4178-16	32.5%	29.9%	52.5%	68.4%	64.0%	67.3%	62.4%	77.3%	75.1%	76.6%	78.3%	78.4%		69.1%	69.5%
AP4144-16	31.2%	29.3%	58.5%	64.3%	64.5%	67.7%	61.9%	69.9%	70.2%	69.3%	70.2%	70.2%	69.1%		99.6%
AP4174-16	32.2%	29.7%	56.2%	63.0%	65.7%	69.0%	60.0%	69.9%	70.7%	70.6%	71.9%	71.8%	69.5%	99.6%	

Fig. 3. Matrix of the nucleotide sequence similarity among the putative new BPV types detected in this study.

1   **Table 1**. Nucleotide sequence similarity of the BPV types detected and gross pattern of the papillomas

2   identified.

BPV type	GenBank accession number	Sample	Similarity	Gross pattern
BPV4	X05817	AP3891-16	98.0%	Flat
BPV6	AJ620208	AP4147-16	98.7%	Flat
		AP4155-16	99.5%	Exophytic
		AP4165-16	99.5%	Flat
		N772-17	100.0%	Flat
BPV7	DQ217793	AP4151-16	99.1%	Exophytic
		AP4159-16	97.3%	Flat
		AP4160-16	99.1%	Flat
		AP4163-16	98.0%	Mixed
BPV8	DQ098913 DQ098917	AP3880-16	99.7%	Mixed
		AP4150-16	99.2%	Mixed
		AP4152-16	97.6%	Flat
		AP4171-16	99.5%	Flat
		AP4834-16	98.8%	Flat
		AP4837-16	99.5%	Flat
		AP768-17	99.6%	Flat
		AP773-17	98.1%	Flat
		AP782-17	89.7%	Flat
		AP3670-16	98.5%	Mixed
		AP3682-16	99.5%	Mixed
		AP3697-16	99.5%	Mixed
BPV9	AB331650	AP3870-16	97.1%	Mixed
BPV10	AB331651 KF017607	AP772-17	98.5%	Mixed
		N770-17	98.9%	Flat
		N771-17	98.7%	Mixed
BPV11	AB543507	AP3898-16	99.1%	Mixed
BPV12	JF834524	AP764-17	90.7%	Mixed

3

1   **Table 2.** Nucleotide sequence similarity between the sequences of the study, theirs closest related

2   Putative BPV types already reported and gross pattern of the papillomas identified.

BPV type	GenBank accession number	Sample	Similarity	Gross pattern
BAPV4	AY426550	AP3694-16	99.3%	Flat
BAPV8	AY426554	AP3689-16	93.1%	Mixed
		AP3696-16	92.5%	Mixed
		AP3895-16	92.6%	Mixed
		AP4176-16	93.7%	Flat
		AP4181-16	92.7%	Mixed
		AP775-17	92.0%	Mixed
BAPV9	AY426555	AP3884-16	99.5%	Mixed
		AP3896-16	99.5%	Mixed
		AP4828-16	98.1%	Flat
		AP778-17	98.8%	Mixed
BPV/BR-UEL2	EU293538	AP3707-16	91.1%	Flat
		AP3888-16	91.1%	Mixed
BPV/BR-UEL5	EU293541	AP4182-16	100.0%	Flat
BPV/CHI-SW2	KF751803	AP3690-16	97.8%	Mixed
		AP3703-16	97.9%	Mixed
		AP4826-16	97.9%	Flat

3

1 **Table 3**. Nucleotide sequence similarity between the putative new BPV types, theirs closest related putative BPV types and BPV types and gross pattern of the

2 papillomas identified.

Sample	BPV type or putative BPV type closest related			BPV type closest related			Gross patern
	Best Blastn Hit	GenBank accession number	Similarity	Best Blastn Hit	GenBank accession number	Similarity	
AP3878-16	BPV/UFPE03BR	JQ897974	79.6%	BPV24	MG602223	73.4%	Mixed
AP3881-16	BPV/UFPE03BR	JQ897974	80.8%	Unclassified	nd	nd	Mixed
AP3885-16	BPV12	JF834524	78.2%	BPV12	JF834524	78.2%	Flat
AP3892-16	BAPV8	AY426554	89.1%	BPV12	JF834524	76.3%	Mixed
AP3899-16	BPV12	JF834524	87.8%	BPV12	JF834524	87.8%	Exophytic
AP4144-16	BPV/BR-UEL3	EU293539	74.3%	BPV9	AB331650	76.3%	Mixed
AP4157-16	BPV/UFPE03BR	JQ897974	80.9%	BPV24	MG602223	75.3%	Flat
AP4167-16	BAA1	AF485375	76.6%	BPV12	JF834524	77.9%	Exophytic
AP4169-16	BPV/UFPE03BR	JQ897974	80.5%	BPV24	MG602223	74.6%	Mixed
AP4174-16	IZ1214/02SP/BR/2009	HQ612180	76.6%	Aks-02	KM983393	80.3%	Mixed
AP4178-16	Aks-02	KM983393	74.6%	Aks-02	KM983393	74.6%	Flat
AP4829-16	BPV/UFPE03BR	JQ897974	80.9%	BPV24	MG602223	75.3%	Flat
AP4835-16	BAPV9	AY426555	83.5%	Unclassified	nd	nd	Mixed
AP771-17	BPV/BR-UEL6	KP892554	76.8%	Unclassified	nd	nd	Mixed
AP781-17	BPV/UFPE03BR	JQ897974	80.1%	BPV24	MG602223	75.5%	Flat

3

4. CONSIDERAÇÕES FINAIS

- Os resultados aqui apresentados permitem concluir que há uma grande variedade de padrões de lesão na glândula mamária de vacas leiteiras, e que esses podem variar de acordo com o agente etiológico envolvido.

- Sete diferentes padrões de mastite foram observados a partir da análise histopatológica (misto, linfoplasmocitário, supurativo, piogranulomatoso, abscedativo, necrossupurativo e granulomatoso).

- *Streptococcus* spp., *Staphylococcus* coagulase negativa (SCN), *Staphylococcus aureus* e *Corynebacterium bovis* foram os principais patógenos associados a casos de mastite em vacas leiteiras abatidas, principalmente correlacionados aos padrões misto, linfoplasmocitário e supurativo.

- *S. aureus* e *Nocardia* sp. foram comumente associados ao padrão piogranulomatoso.

- *Trueperella pyogenes* foi correlacionada a quase todos os casos de mastite abscedativa, assim como SCN e *Escherichia coli* ao padrão necrossupurativo.

- As lesões papilomatosas de tetos apresentaram três padrões macroscópicos (exofítico, plano e misto); todos caracterizados por papilomas escamosos na histopatologia.

- Diferentes tipos de papilomavírus bovino (BPV) foram identificados, sendo oito tipos clássicos de BPV, seis prováveis tipos de BPV previamente descritos e 10 prováveis novos tipos de BPV.

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