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CARCINOMAS DE CÉLULAS ESCAMOSAS DO TRATO ALIMENTAR SUPERIOR DE BOVINOS ASSOCIADOS À INTOXICAÇÃO CRÔNICA POR SAMAMBAIA (*Pteridium arachnoideum*)

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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Patologia e Patologia Clínica Veterinária, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do título de **Doutor em Medicina Veterinária**

Orientadora: Prof[a] Dr[a]. Glaucia Denise Kommers

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RESUMO

CARCINOMAS DE CÉLULAS ESCAMOSAS DO TRATO ALIMENTAR SUPERIOR DE BOVINOS ASSOCIADOS À INTOXICAÇÃO CRÔNICA POR SAMAMBAIA (*Pteridium arachnoideum*)

AUTORA: Tatiane Cargnin Faccin
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Os neoplasmas do trato alimentar superior (TAS) são raros em bovinos, porém em algumas regiões, como no Sul do Brasil, os carcinomas de células escamosas (CCEs) do TAS são relativamente comuns e estão associados à ocorrência concomitante de papilomas e ao consumo de samambaia. Embora haja uma teoria de patogênese que considera o papilomavírus bovino tipo 4 (BPV-4) como um co-fator no desenvolvimento desses CCEs, ainda há aspectos da etiopatogênese dessa condição a serem investigados, pois a detecção do BPV-4 em outras regiões geográficas, além das poucas previamente publicadas, é escassa. Além disso, no Brasil, os CCEs esofágicos são observados com uma apresentação macroscópica incomum, como um espessamento anelar estenosante da parede esofágica, que é diferente das formas comuns de apresentação deste tumor que são massas exofíticas ou endofíticas ulceradas ou infiltrativas. Portanto, os objetivos desta tese foram (1) analisar os aspectos epidemiológicos, clínicos e patológicos de 100 casos de CCEs do TAS em bovinos que pastejavam em áreas contaminadas por samambaia (*Pteridium arachnoideum*), bem como investigar as associações entre esses parâmetros; (2) investigar a presença de papilomavírus nos papilomas do TAS por reação em cadeia da polimerase (PCR) e por imuno-histoquímica; (3) e detalhar os aspectos clínicos e patológicos de 13 casos de CCEs esofágicos anelares estenosantes. No primeiro estudo, sobre os 100 casos de CCEs no TAS, associações estatisticamente significativas entre os sinais clínicos e a localização do tumor no TAS, entre o grau de diferenciação histológica e a localização do tumor e uma tendência a associação significativa entre o grau de diferenciação histológica e a presença de metástases foram observadas. A média de idade dos bovinos com CCEs orofaríngeos foi 7,39 anos, com diferença significativa quando comparado com bovinos com neoplasmas esofágicos (8,6 anos). DNA ou antígeno de papilomavírus não foram detectados nos papilomas por PCR e imuno-histoquímica, respectivamente. Desta forma, os resultados mantem aberta a possibilidade de que papilomas de TAS podem não estar associados ao papilomavírus. Assim, a infecção por BPV-4 pode não ser um co-fator no desenvolvimento dos CCEs em bovinos da região estudada. No segundo estudo, sobre os CCEs esofágicos anelares estenosantes, massas endofíticas, circunferenciais (anelares) no esôfago, com estreitamento luminal (estenose) foram observadas. Os ceratinócitos neoplásicos eram circundados por tecido conjuntivo fibroso moderado a abundante (reação desmoplástica). A coloração de Picrosírius sob luz polarizada demonstrou fibras de colágeno tipo I abundantes, que contribuiram para as características estenosantes deste tumor. Esses CCEs esofágicos devem ser incluídos no diagnóstico diferencial de estenose esofágica.

Palavras-chave: Doenças de bovinos. Neoplasmas do trato digestório. *Pteridium* spp. Samambaia. Carcinoma de células escamosas. Papilomas.

ABSTRACT

SQUAMOUS CELL CARCINOMAS OF THE UPPER ALIMENTARY TRACT OF CATTLE ASSOCIATED WITH CHRONIC POISONING BY BRACKEN FERN (*Pteridium arachnoideum*)

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Upper digestive tract (UDT) neoplasms are rare in cattle, but in some regions, such as Southern Brazil, UDT squamous cell carcinomas (SCCs) are relatively common and have been associated with the concomitant occurrence of papillomas and the consumption of bracken fern. Although there is a theory of pathogenesis that considers bovine papillomavirus type 4 (BPV-4) as a co-factor in the development of these SCCs, some aspects of the etiopathogenesis of this condition need to be investigated, since the detection of BPV-4 in other geographic regions, in addition to the few ones previously published, is scarce. Additionally, esophageal SCCs in Brazil are observed with an uncommon gross presentation, such as an annular stenotic thickening of the esophageal wall, which is different from the usual forms of presentation of this tumor, which are exophytic or ulcerative or infiltrative endophytic neoplasms. Therefore, the aims of this thesis were (1) to analyze the epidemiological, clinical and pathological aspects of 100 cases of SCCs in the UDT of cattle grazing on bracken fern (*Pteridium arachnoideum*) highly contaminated areas, as well as to investigate associations between these parameters; (2) to investigate the presence of papillomavirus in the UDT papillomas by polymerase chain reaction (PCR) and by immunohistochemistry; (3) and to elucidate the clinical and pathological aspects of annular stenotic esophageal SCCs. In the first paper, about 100 cases of SCCs of the UDT, there were statistically significant associations between clinical signs and tumor localization in the UDT, between histological grade of differentiation and tumor localization, and a trend towards significant association between histological grade of differentiation and presence of metastases. The average age of cattle with oropharyngeal SCCs was 7.39 years old, with significant difference compared to cattle with esophageal SCCs (8.6 years). No papillomaviral DNA or antigen were detected in papillomas by PCR and immunohistochemistry, respectively. Therefore, these results keep open the possibility that papillomas of the UDT may not be associated with papillomavirus. Thus, the BPV-4 infection may not be a co-factor to the development the SCCs in cattle from the region studied. In the second paper, about annular stenotic esophageal SCCs, endophytic masses, circumferential (annular) within the esophageal with luminal narrowing (stenosis) were observed. The neoplastic keratinocytes were surrounded by moderate to abundant fibrous connective tissue (desmoplastic reaction). Picrosirius red-stained sections under polarized light showed abundant collagen type I fibers, which contributed to the stenosing characteristics of this tumor. These esophageal SCCs should be included in the differential diagnosis of esophageal stenosis.

Keywords: Diseases of cattle. Neoplasms of the digestive tract. *Pteridium* spp. Bracken fern. Squamous cell carcinoma. Papillomas.

SUMÁRIO

1 INTRODUÇÃO

A samambaia (*Pteridium* spp., Dennstaedtiaceae) (THOMSON, 2000) é considerada uma das plantas tóxicas mais importantes, não só por ser cosmopolita e ter extensa distribuição, mas também pelos diferentes tipos de intoxicação que provoca em diversas espécies animais (TOKARNIA et al., 2012). A intoxicação por samambaia é de grande importância em muitos Estados do Brasil. *Pteridium arachnoideum* é a principal causa de intoxicação por plantas em bovinos em Santa Catarina (BORELLI et al., 2008) e é uma causa importante de intoxicação em bovinos na região Central do Rio Grande do Sul (RS) (MASUDA et al., 2011a; RISSI et al., 2007; SOUTO et al., 2006a), Paraná (MARÇAL, 2003) e nas regiões Sudeste (SILVA et al., 2009) e Centro-Oeste (FURLAN et al., 2014a, b).

Além de carcinomas de células escamosas (CCEs) no trato alimentar superior (TAS) (SOUTO et al., 2006a), a intoxicação crônica por samambaia também pode causar hematúria enzoótica bovina, caracterizada por hematúria e neoplasmas na vesícula urinária (FURLAN et al., 2014a; GABRIEL et al., 2009; SOUTO et al., 2006b) e uma síndrome hemorrágica aguda, associada à aplasia de medula óssea (ANJOS et al., 2008, 2009; FURLAN et al., 2014b). Na região Central do RS, na área de abrangência do Laboratório de Patologia Veterinária da Universidade Federal de Santa Maria (LPV-UFSM), as formas de intoxicação crônica, principalmente a caracterizada por CCEs no TAS, têm sido estudadas sob vários aspectos. Os aspectos epidemiológicos, clínico-patológicos (GABRIEL et al., 2009; SOUTO et al., 2006a) e morfológicos (MASUDA et al., 2011a, b; SOUTO et al., 2006a) foram abordados em outras publicações. Porém, aspectos associados à etiopatogênese ainda não foram avaliados na nossa região, como por exemplo, a investigação da presença do papilomavírus bovino tipo 4 (BPV-4) nas lesões papilomatosas pela técnica de reação em cadeia da polimerase (PCR). Deve-se levar em conta que, no Brasil, não foram encontradas publicações confirmando que os papilomas vistos no TAS de bovinos realmente são consequentes da infecção pelo BPV-4. A técnica de imuno-histoquímica (IHQ) também não foi utilizada na detecção de antígenos virais de papilomavírus (PV) em estudos de intoxicação crônica por samambaia com papilomatose alimentar concomitante.

Um estudo amplo, compreendendo um expressivo número de casos desta forma da intoxicação, remetidos ao LPV-UFSM (período de 2003 a 2014), permitirá verificar a distribuição das lesões (carcinomatosas e papilomatosas) e a ocorrência e distribuição das metástases. Permitirá também detalhar fatores clínicos e patológicos da apresentação macroscópica de CCEs caracterizados por espessamentos anelares estenosantes esofágicos

observados nessa forma da intoxicação crônica por samambaia, que não têm sido relatados em outros animais domésticos (HEAD; ELSE; DUBIELZIG, 2002) ou em humanos (GABBERT et al., 2000).

Desta forma, os objetivos desta tese foram estudar os CCEs do TAS em bovinos associados à ingestão crônica por samambaia recebidos no LPV-UFSM; analisar os aspectos epidemiológicos, clínicos e patológicos dessa condição, bem como investigar as associações entre esses parâmetros; investigar a presença de DNA de PV nos papilomas do TAS por PCR e de antígenos virais de PV por IHQ; e elucidar os aspectos clínicos e patológicos de CCEs esofágicos anelares estenosantes.

2 REVISÃO DE LITERATURA

2.1 ASPECTOS GERAIS DA SAMAMBAIA (*Pteridium* spp.)

A samambaia é uma planta tóxica, cosmopolita e invasora de pastagens (TOKARNIA et al., 2012). Até recentemente, o gênero *Pteridium* era constituído de apenas uma espécie (*P. aquilinum*) e de cinco subespécies; essas subespécies foram elevadas a espécies, das quais duas existem no Brasil (*P. arachnoideum* e *P. caudatum*). *P. arachnoideum* (Kaulf.) Maxon ocorre em regiões montanhosas e se desenvolve melhor em regiões frias e de boa pluviosidade, com solos ácidos e bem drenados, como encostas de morros e é encontrada nas regiões Norte (PA, AM), Nordeste (CE, PE, BA, AL), Centro-Oeste (DF, MS, MT), Sudeste (MG, ES, SP, RJ) e Sul (PR, SC, RS) do Brasil, assim como na Amazônia, Cerrado, Mata Atlântica, Pampa e Pantanal, enquanto que *P. caudatum* (L.) Maxon tem sido observada nas regiões Norte (AM, AC, RO), Centro-Oeste (MT) e Nordeste e também na Amazônia (FURLAN et al., 2014b; PRADO; SYLVESTRE, 2010; TOKARNIA et al., 2012).

O impacto negativo causado pelas intoxicações por plantas na pecuária justifica o desenvolvimento nas últimas décadas de um grande número de pesquisas para caracterizar a epidemiologia e desenvolver tecnologias de controle e profilaxia dessas intoxicações (PESSOA; MEDEIROS; RIET-CORREA, 2013). Alguns fatores têm sido implicados na invasão das pastagens por samambaia. A utilização de áreas exclusivamente para a pecuária tem sido considerada um fator importante para o desenvolvimento da planta. Em um estudo realizado no Estado do Mato Grosso, em todos os estabelecimentos nos quais se constatou a presença da planta, as áreas invadidas eram utilizadas exclusivamente para a pecuária. *Pteridium* spp. não foram observadas em áreas utilizadas para agricultura ou em áreas em que se que realizava integração lavoura-pecuária, mesmo quando estas faziam divisa com piquetes invadidos pela planta. Áreas antes utilizadas como pastagem e que eram severamente invadidas por *Pteridium* spp., não apresentaram mais invasão pela planta quando passaram a ser utilizadas para o plantio de soja (FURLAN et al., 2014b). Isso ocorre possivelmente devido à correção do solo realizada frequentemente nestes sistemas, uma vez que a planta se desenvolve melhor em áreas de solos ácidos (TOKARNIA et al., 2012), ou ainda a outras práticas agrícolas utilizadas nesses sistemas como a lavração, a fertilização e o plantio de outras culturas que lhe tiram a luz (FURLAN et al., 2014b).

Outro aspecto importante para o desenvolvimento da planta são as queimadas. Observou-se que uma pequena quantidade da planta nas pastagens pode se alastrar pelos

piquetes, muitas vezes chegando a se tornar a vegetação predominante, após a realização de queimadas. Além disso, outro fator observado em muitas propriedades no Mato Grosso foi a crescente expansão das lavouras de soja, desse modo, áreas antes utilizadas como pastagens deram lugar à agricultura e novas áreas foram desmatadas para alojar o gado. Nessas novas áreas *Pteridium* spp. têm se alastrado vigorosamente, principalmente após as queimadas (FURLAN et al., 2014b). Segundo Alonso-Amelot (1999), em regiões fechadas de florestas tropicais, a planta não ocorre por encontrar condições incompatíveis com seu desenvolvimento, pois seu crescimento é favorecido quando em plena exposição à luz (MARRS; WATT, 2006), como ocorre em áreas recém-desmatadas e após a utilização de queimadas.

2.2 ETIOPATOGÊNESE DE CCEs E DE PAPILOMAS DO TAS DE BOVINOS

Os CCEs são neoplasmas epiteliais malignos de células escamosas. Este é um dos tumores malignos de pele mais comuns dos animais domésticos, incluindo as aves. Existem vários fatores que estão associados ao desenvolvimento de CCEs cutâneos, incluindo exposição prolongada à luz ultravioleta, pele despigmentada e ausência ou pequena quantidade de pelo nos locais afetados. Portanto, a localização geográfica e o clima (exposição à luz ultravioleta) e a localização anatômica (conjuntiva, vulva, períneo) podem influenciar significativamente a incidência deste tipo de câncer (GOLDSCHMIDT; GOLDSCHMIDT, 2017).

As células escamosas também revestem o sistema digestório; porém em bovinos, a ocorrência de CCEs no TAS é rara; exceto em alguns países, como Brasil (SOUTO et al., 2006a; TOKARNIA; DÖBEREINER; CANELLA, 1969), Escócia (JARRETT et al., 1978) e Itália (BORZACCHIELLO et al., 2003) onde este tipo de tumor é relativamente comum e está associado com papilomas e intoxicação crônica por samambaia.

Os papilomas são neoplasmas benignos do epitélio que possuem etiologia e patogênese complexas (KUMAR; ABBAS; ASTER, 2015). A maioria dos papilomas são causados por infecções por PV; porém nem todos os papilomas são causados ou contêm vírus (GOLDSCHMIDT; GOLDSCHMIDT, 2017; MAULDIN; PETERS-KENNEDY, 2016). Em bovinos, o BPV-4, da família *Papillomaviridae*, gênero *Xipapillomavirus*, espécie *Xipapillomavirus 1* (INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES, 2017; LOVATO, 2007), infecta a mucosa do TAS e leva à formação de papilomas que regridem naturalmente (JARRETT, 1980), pois são auto-limitantes (CAMPO et al., 1994). No entanto, em regiões com grande quantidade por samambaia, a presença e a persistência de acentuada quantidade de papilomas do TAS (papilomatose) é comum em bovinos de diferentes idades

(DÖBEREINER; TOKARNIA; CANELLA, 1967; MASUDA et al., 2011b; SOUTO et al., 2006a) e há uma teoria de que a samambaia esteja implicada na manutenção dos papilomas no TAS, por causar imunossupressão e na transformação dos mesmos em CCEs (CAMPO et al., 1994).

PV são vírus DNA de cadeia dupla, circulares, não envelopados e epiteliotrópicos (ALFIERI; ALFIERI; WOSIACKI, 2007; INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES, 2017). Os PV podem ser associados a papilomas e a tumores malignos como CCEs de colo do útero, vagina, pênis, ânus e cavidade oral em humanos (GILLISON et al., 2000; PARKIN, 2006; zur HAUSEN, 2009) e câncer de vesícula urinária e TAS em bovinos (GABRIEL et al., 2009; MASUDA et al., 2011a; SOUTO et al., 2006a, b). Estima-se que os PV de mucosa causem 5,2% de todos os cânceres humanos. A maioria dos PV são altamente espécie-específicos (de VILLIERS et al., 2004). Cada espécie pode ser infectada por vários PV, com cada subtipo de vírus frequentemente associado a um tecido específico (GOLDSCHMIDT; GOLDSCHMIDT, 2017). Em humanos, fortes evidências indicam que alguns tipos de PV da mucosa causam neoplasia genital e oral, e permanece incerto se alguns PV cutâneos podem causar CCEs cutâneos (AKGUL; COOKE; STOREY, 2006; MEYER et al., 2001; zur HAUSEN, 2009). Para as espécies não-humanas, fortes evidências apoiam o papel causal dos PV no desenvolvimento de sarcoides felinos e equinos. Do mesmo modo, acredita-se que os PV causem CCEs cutâneos em coelhos, marsupiais *(Perameles bougainville)* e alguns roedores. Além disso, algumas evidências sugerem que os PVs podem influenciar o desenvolvimento de CCEs cutâneos felinos e caninos (MUNDAY; KIUPEL, 2010).

Ao considerar a infecção da epiderme por PV, deve-se considerar a estratificação dos ceratinócitos em uma população de células basais auto-renováveis e uma população de ceratinócitos diferenciados terminais (ALONSO; FUCHS, 2003). A infecção das células basais por PV pode resultar em uma infecção assintomática persistente. No entanto, para que a replicação viral ocorra, as células basais infectadas devem se tornar terminalmente diferenciadas (ORTH, 2006). Portanto, para maximizar a replicação viral, os PV tentam aumentar a proliferação de células basais e a diferenciação de ceratinócitos terminais (MÜNGER et al., 2004). Se bem sucedido, os PV estimulam a proliferação epidérmica que pode resultar no dobramento da epiderme e no desenvolvimento de um papiloma viral (de VILLIERS et al., 2004). O desenvolvimento de uma resposta imune provoca a resolução da maioria dos papilomas virais (GROSS et al., 2005). Os PV podem influenciar o crescimento e

diferenciação celular; como tal, eles podem ter o potencial de promover a transformação neoplásica de células infectadas (MUNDAY; KIUPEL, 2010).

Em bovinos, 13 PV têm sido descritos. A papilomatose bovina pode ser um problema de rebanho na medida em que o vírus é facilmente transmitido por contato animal-animal e por fômites (MAULDIN; PETERS-KENNEDY, 2016). Dependendo do sítio anatômico e do tipo de PV, ambas as características morfológicas e biológicas das lesões podem ser diferentes, conforme apresentado no Quadro 1.

Quadro 1 – Papilomaviroses dos bovinos

Vírus	**Gênero do PV**	**Lesão**
BPV-1	Delta	Fibropapiloma genital, ruminal, cutâneo e no teto; papiloma ruminal; neoplasma na vesícula urinária; sarcoide equino
BPV-2	Delta	Fibropapiloma genital, ruminal, cutâneo e no teto; papiloma ruminal; neoplasma da vesícula urinária; sarcoide equino
BPV-3	Xi	Papiloma cutâneo
BPV-4	Xi	Papiloma oral, esofágico e ruminal; neoplasma na vesícula urinária
BPV-5	Epsilon	Fibropapiloma ruminal e no teto; papiloma ruminal e cutâneo
BPV-6	Xi	Papiloma no teto
BPV-7	Dyoxi	Papiloma no teto; pele saudável
BPV-8	Epsilon	Papiloma cutâneo, fibropapiloma
BPV-9	Xi	Papiloma no teto
BPV-10	Xi	Papiloma no teto, papiloma na língua
BPV-11	Xi	Papiloma cutâneo
BPV-12	Xi	Papiloma na língua
BPV-13	Delta	Papiloma na orelha e sarcoide equino

Fonte: Traduzido de Mauldin; Peters-Kennedy (2016).

Alguns BPV estão associados à transformação neoplásica na pele, no trato digestório e na vesícula urinária. Muitos fatores estão envolvidos, incluindo proteínas virais que promovem a desregulação celular e a evasão imune. Em conjunto com vários BPV (BPV-1, BPV-2 e BPV-4), a ingestão de samambaia tem sido associada a várias neoplasias epiteliais e mesenquimais da vesícula urinária e do TAS (MAULDIN; PETERS-KENNEDY, 2016).

Após tentativa com pequeno sucesso de detecção do DNA viral em um número significativo de amostras de CCEs no TAS de bovinos pela técnica de hibridização por *Southern blot*, os autores concluíram que, embora o BPV-4 seja o agente etiológico da papilomatose

alimentar bovina, a presença do DNA viral não seria necessária para a progressão ou para a manutenção do estado transformado, ou seja, para a transformação maligna dos ceratinócitos (CAMPO et al., 1985). O papel do BPV-4 na carcinogênese estaria confinado aos primeiros estágios de transformação celular, e à proliferação celular induzida pelo BPV-4 proveria um alvo amplificado para a ação dos carcinógenos químicos presentes na samambaia (CAMPO et al., 1994). Todavia, a ação direta da carcinogênese química do principal princípio tóxico da samambaia, o ptaquilosídeo norsesquiterpeno, sem um envolvimento viral, já foi amplamente observada em vários estudos (EVANS et al., 1961; FENWICK, 1988; HIRONO et al., 1973). A marcada presença de lesões intraepiteliais escamosas (SILs) e de CCEs invasivos em estágio inicial apontam para outro caminho no desenvolvimento de CCEs do TAS em bovinos com intoxicação crônica por samambaia. Embora um papel das oncoproteínas do BPV-4 no desenvolvimento das SILs (com grande probabilidade de progressão para CCEs) não pode ser desconsiderado, os resultados podem indicar que a ação direta dos carcinógenos da samambaia podem representar uma rota alternativa de etiopatoênese para o desenvolvimento de CCEs em bovinos (MASUDA, 2010).

Ao mesmo tempo em que há evidências de estudos *in vitro* de que a infecção pelo BPV-4 predispõe a transformação neoplásica (BENISTON et al., 2001), a ausência do DNA do BPV-4 nos CCEs do TAS de bovinos e a necessidade da samambaia para a ocorrência dessa neoplasia torna difícil determinar o papel preciso do BPV-4 no desenvolvimento desse neoplasma maligno. Enquanto o BPV-4 parece ser um importante cofator no desenvolvimento neoplásico, não pode ser excluída a possibilidade de que papilomas e carcinomas sejam ambos independentemente induzidos pela samambaia (MUNDAY, 2014). Os estudos experimentais em ratos têm demonstrado que o ptaquilosídeo sozinho é cancerígeno (HIRONO et al., 1987).

2.3 EPIDEMIOLOGIA E SINAIS CLÍNICOS DA INTOXICAÇÃO CRÔNICA POR SAMAMBAIA NA FORMA DE CCEs NO TAS

A ocorrência dos CCEs no TAS está associada a ingestão crônica de samambaia no Brasil (DÖBEREINER; TOKARNIA; CANELLA, 1967; GAVA et al., 2002; MASUDA et al., 2011a; SOUTO et al., 2006a; TOKARNIA; DÖBEREINER; CANELLA, 1969) e em outros países (BORZACCHIELLO et al., 2003; JARRETT et al., 1978).

A fome é o principal fator associado à ingestão da planta (FURLAN et al., 2014b) e está relacionada à escassez de alimento devido a fatores como a superlotação ou períodos de seca.

Isto normalmente ocorre, pois, a planta suporta bem o período sem chuvas, possibilitando sua procura pelos animais (MARÇAL, 2003).

Tem sido sugerido que o vício também pode condicionar a ingestão visto que, após consumir a planta durante certo período de tempo, os bovinos desenvolvem o hábito de ingeri-la mesmo havendo disponibilidade de alimento (TOKARNIA et al., 2012). O terceiro fator implicado na procura dos animais pela planta é a carência de pastagem fibrosa (MARÇAL, 2003; TOKARNIA et al., 2012). Como a samambaia costuma se desenvolver e atingir boa altura, os bovinos suprem a necessidade de fibra, comendo os caules e folhas longas que a planta normalmente possui (TOKARNIA et al., 2012). A ingestão de feno contaminado ou de cama que contenha samambaia pode contribuir para a ocorrência da intoxicação (MARÇAL, 2003). A intoxicação também tem sido observada em animais consumindo os rizomas expostos após a aração (TOKARNIA et al., 2012).

Outro fator importante relacionado à epidemiologia dessa intoxicação é o período de ingestão da planta. Os bovinos têm que ingerir samambaia por períodos mais ou menos prolongados para que a intoxicação ocorra (PESSOA; MEDEIROS; RIET-CORREA, 2013; TOKARNIA et al., 2012). A hematúria enzoótica afeta bovinos com idade a partir de dois anos, que ingerem quantidades menores que 10g/kg de peso vivo por dia da planta durante um ou mais anos. Acredita-se que as quantidades diárias de samambaia necessárias para que ocorra a forma de CCEs no TAS sejam ainda menores do que aquelas necessárias para causar o quadro de hematúria enzoótica bovina, sendo que a ingestão da planta ocorreria durante um período de tempo mais prolongado (TOKARNIA et al., 2012).

Em estudo realizado na região Central do Estado do RS, a taxa de morbidade foi de 2,7% numa população de 1090 bovinos. A letalidade foi considerada como de 100% (SOUTO et al., 2006a). Esses dados assemelham-se aos descritos na literatura (morbidade de 3% e letalidade de 100%) para essa forma de intoxicação no Estado de Santa Catarina (GAVA, 1993).

A idade dos bovinos intoxicados pode variar entre três e 13 anos, sendo o maior número de casos observados entre sete e oito anos (46,6%) (SOUTO et al., 2006a). Outros autores destacam uma maior casuística em bovinos acima de seis anos (TOKARNIA et al., 2012). Em outros países, a idade dos animais variou de 7 a 18 anos (JARRETT et al., 1978).

Não há predisposição por raça ou sexo (SOUTO et al., 2006a), porém, as fêmeas parecem ser mais afetadas devido à maior permanência desses animais na fazenda. Pela mesma razão, os bois destinados ao trabalho também podem ser mais afetados por CCEs no TAS (SOUTO et al., 2006a).

Os sinais clínicos descritos nessa forma de intoxicação são tosse, ronqueira, dificuldade respiratória, corrimento com fragmentos alimentares pelas narinas e boca, dificuldade de mastigação, deglutição e ruminação, atonial ruminal, diarreia, regurgitação de alimento, timpanismo crônico intermitente, emagrecimento e morte (DÖBEREINER; TOKARNIA; CANELLA., 1967; SOUTO et al., 2006a; TOKARNIA; DÖBEREINER; CANELLA, 1969). Os sinais clínicos são dependentes da localização do CCE no TAS e, na grande maioria dos casos, estão associados ao CCE de maior tamanho (SOUTO et al., 2006a).

2.4 MORFOLOGIA DOS CCEs NO TAS NA INTOXICAÇÃO CRÔNICA POR SAMAMBAIA

A distribuição dos CCEs no TAS não é regular e diferentes percentuais de frequência de aparecimento deste tumor têm sido observados em diferentes estudos (GAVA et al., 2002; JARRET et al., 1978; SOUTO et al., 2006a). Na região Central do RS, em estudos com 30 (SOUTO et al., 2006a) e 40 (MASUDA et al., 2011a) casos analisados, os CCEs acometeram principalmente as regiões cranial (base da língua, faringe/orofaringe e epiglote) e caudal (entrada do rúmen) do TAS, sendo menos frequentes no esôfago. Diferentemente de outros países, onde os CCEs esofágicos foram mais frequentes (JARRETT et al., 1978).

Os CCEs alimentares em bovinos se apresentam macroscopicamente como massas tumorais exuberantes e de crescimento exofítico, áreas rugosas de crescimento endofítico, ou de espessamentos anelares estenosantes (esses últimos observados somente na parede esofágica) (MASUDA et al., 2011a; SOUTO et al., 2006a). Esta apresentação incomum de espessamento anelar dos CCEs esofágicos tem sido mencionada desde os primeiros relatos da doença em bovinos (HEAD; ELSE; DUBIELZIG, 2002; LUCENA et al., 2011; MASUDA et al., 2011a; SOUTO et al., 2006a; TOKARNIA; DÖBEREINER; CANELLA, 1969), sem estudos adicionais sobre seus aspectos clínicos e patológicos particulares. Os CCEs normalmente variam entre 8-50 cm, podendo ser sésseis ou pedunculados, com extensas áreas de ulceração ou de necrose. Na superfície de corte, pontos ou áreas irregulares, amareladas foram vistas onde havia acentuada ceratinização. Áreas firmes e brancas também foram observadas e associadas à ocorrência de desmoplasia (MASUDA et al., 2011a; SOUTO et al., 2006a,). A frequência do padrão de crescimento desses neoplasmas não foi relatada.

A ocorrência de metástases foi observada em 36% (JARRET et al., 1978), 58% (MASUDA et al., 2011a) ou em 60% dos casos (SOUTO et al., 2006a), e os linfonodos regionais eram os mais acometidos (MASUDA et al., 2011a). Metástases para o fígado,

pulmões e baço foram raras e sempre associadas com CCEs na entrada do rúmen. Metástases para os linfonodos regionais e / ou órgãos distantes foram encontrados em 44,5% dos tumores bem diferenciados, 75% dos tumores moderadamente diferenciados e todos tumores pouco diferenciados em um estudo de 40 casos (MASUDA et al., 2011a). Esses dados demonstraram que o grau de diferenciação do tumor é importante para ocorrência de metástases. Outros fatores, implicados na progressão tumoral têm sido estudados, como padrão de migração e invasão celular, infiltrado linfoplasmocítico e eosinofílico e desmoplasia, dentre outros (MASUDA et al., 2011a).

3 ARTIGO 1 - BOVINE UPPER ALIMENTARY SQUAMOUS CELL CARCINOMA ASSOCIATED WITH BRACKEN FERN POISONING: CLINICAL-PATHOLOGICAL ASPECTS AND ETIOPATHOGENESIS OF 100 CASES

Artigo a ser submetido para PLOS ONE.

Bovine upper alimentary squamous cell carcinoma associated with bracken fern poisoning: clinical-pathological aspects and etiopathogenesis of 100 cases

Short tittle: Bracken fern-associated cancer in cattle

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Abstract

Upper digestive tract (UDT) cancer is rare in cattle, however in Southern Brazil, the UDT squamous cell carcinomas (SCCs) are relatively common and have been associated with bracken fern consumption and the presence of papillomas. Although a theory of pathogenesis considers bovine papillomavirus type 4 (BPV-4) as a cofactor in the development of these SCCs, some aspects of the etiopathogenesis of this disease need to be more investigated. In fact, detection of BPV-4 in UDT papillomas in other geographic regions, in addition to the few ones previously published, is scarce. Therefore, this study aimed to analyze the epidemiological, clinical and pathological aspects of 100 natural cases of SCCs in the UDT of cattle grazing on bracken fern (*Pteridium arachnoideum*) highly contaminated areas. The associations between these parameters were investigated as well as the presence of papillomavirus in the UDT papillomas by polymerase chain reaction (PCR, n=47) and immunohistochemistry (n=93). There were statistically significant associations between clinical signs and tumor localization in the UDT; between histological grade of differentiation and tumor localization; and a trend towards significant association between histological grade of differentiation and presence of metastases. The average age of cattle with oropharyngeal SCCs was 7.39 years, with statistically significant difference comparing to cattle with esophageal SCCs (8.6 years). No statistical association was observed among other clinical-pathological parameters (sex, growth pattern, primary site of the tumor) analyzed. No BPV DNA or antigens were detected in papillomas by PCR and immunohistochemistry, respectively. Therefore, these results suggest the possibility that papillomas of the UDT are not necessarily associated with BPV infection.

Introduction

Squamous cell carcinomas (SCCs) are the most common oral neoplasms of humans, cats, horses, and livestock species and are the second most common of dogs, behind only the melanocytic tumors [1-4]. Oral neoplasms are generally rare in livestock [3, 5]. An exception is seen in a few geographic areas, such as Southern Brazil, where oral, pharyngeal, esophageal, and ruminal SCCs may represent around 20% of all neoplasia of cattle [6]. In these regions, SCCs of the upper digestive tract (UDT) are associated with chronic bracken fern (*Pteridium* spp.) poisoning and presence of UDT papillomas [3, 5, 7-9].

Some studies show that UDT papillomas are caused by a productive bovine papillomavirus type 4 (BPV-4) infection [10, 11]. Although histological and electron microscopic data suggested a role of BPV-4 in the development of alimentary SCCs [7], and the disease has been experimentally reproduced [12], no BPV-4 DNA could be detected in the SCCs by Southern blot hybridization [13] and studies using a more sensitive technique as polymerase chain reaction (PCR) have not been performed.

In Brazil, the relationship between these neoplasms and chronic ingestion of bracken fern is well established [6, 8, 9, 14], but there are no studies confirming the association of BPV-4 with this condition. Experimental reproduction of the disease is difficult to perform because cattle need to consume small amounts of the plant for several years to develop SCCs [15].

Therefore, the aims of this study were to analyze the epidemiological, clinical and pathological aspects of 100 natural cases of SCCs in the UDT of cattle grazing for years on bracken fern (*Pteridium arachnoideum*) highly contaminated areas in Southern Brazil. The associations between these parameters and the presence of

papillomavirus in the UDT papillomas by PCR and immunohistochemistry were also investigated.

Material and methods

Tumors from 100 cattle with SCCs in the UDT were collected between September 2003 and August 2014. Samples were obtained from necropsy examinations performed by veterinary pathologists in our laboratory or by a trained field veterinarian, who submitted the whole UDT (from the tongue to the rumen) in all suspected cases of chronic poisoning by bracken fern. Age, sex, breed, clinical signs, tumor growth pattern (endophytic or exophytic) and the frequency and localization of metastases were recorded. The localization of the tumors was classified as oropharyngeal (including base of the tongue, pharynx and epiglottis), esophageal (proximal, middle and distal esophagus) or ruminal (rumen entrance), according to the tumor primary sites. When cattle had more than one SCC in the UDT, the largest tumor in centimeters was considered for this study [9]. The growth pattern of the cancer was determined by visual examination with careful palpation on natural and cut surfaces.

Samples of the lesions were fixed in 10% buffered formalin, routinely processed for histopathology and stained with hematoxylin and eosin. Microscopically, each SCC was graded as well, moderately, or poorly differentiated [14, 16].

Immunohistochemistry

Immunohistochemical staining was performed in 93 papillomas of 38 cattle with a human papillomavirus (HPV) cocktail broad spectrum (HPV-1, 6, 11, 16, 18 and 31), clone BPV-1/1H8 + CAMVIR-1, mouse monoclonal antibody (Biocare Medical, Pacheco, CA, USA). Slides were placed in 3% hydrogen peroxide for endogenous

peroxidase blockage. Antigen retrieval was performed by pretreating with microwave heating for 10 min in Tris–EDTA buffer pH 9.0. Nonspecific reactions were blocked with protein blocker (EasyPath, Erviegas Ltda, São Paulo, Brazil) for 10 min. The primary antibody was applied at a concentration of 1:100 for 30 min at room temperature. A peroxidase-polymer (EasyLink One, EasyPath) was then applied for 20 min. DAB substrate chromogen (EasyPath) was applied to the slides for 7 min. Sections were counterstained with Harris hematoxylin. Positive control specimens included tissues of oral canine papillomatosis and bovine cutaneous papillomatosis. As negative controls, the primary antibody was replaced with PBST. Strong nuclear immunoreactivity was present primarily within the superficial keratinocytes in positive controls.

DNA extraction and PCR

Frozen (n=42) and fresh (n=5) fragments (50-100mg) of papillomas collected from 30 bovine oral or esophageal mucosa were submitted to total DNA extraction using the phenol and chloroform method. The samples were lysed and digested using 1x sodium dodecyl sulfate solution and 1mg/ml of proteinase K. The samples were incubated during 1-3h at 56°C. The organic and DNA phases were separated using phenol and chloroform solution and centrifugation (9,000 rpm, 30min). DNA precipitation was performed using ethanol and total DNA was eluted in 100µl of Tris-EDTA solution.

Total DNA was submitted to a PCR to amplify a 165 bp segment of the BPV-4 L1 gene. Initially, the sensitivity of the PCR was analyzed using the primers described by Borzacchiello et al. [11]. The positive control was a synthetic DNA containing the target sequence, that contains approx. 20 nucleotides plus flanking regions. The

synthetic DNA was constructed using the complete sequence of BPV-4 deposited in Genbank (accession number X05817.1) as the model. This PCR allowed for the amplification of as low as 1.56x 10^6 DNA copies. PCR reactions were performed in 25µl volume, using 2µl of template DNA, 12.5µM of each primer, 2.5mM of $MgCl_2$, 10mM of dNTPs, 1x reaction buffer and 1 unit of Taq DNA polymerase (ThermoFischer Scientific®). PCR conditions were: initial denaturation (95°C for 10 min), followed by 30 cycles of 95°C - 60s; 45°C - 60s for primer annealing and 72°C - 60s for primer extension; and a final extension of 7 min at 72°C. Products were visualized in a 1.5% agarose gel, stained with Gel Red (Biotium, Inc., Fremont, CA) and visualized under UV light.

Panpapillomavirus PCR was also performed, using primers FAP59/64 and conditions described by Forslund et al. [17], and DNA of bovine papillomavirus type 1 extracted of a cutaneous wart was used as positive control. All reactions were performed using ultrapure water as negative control.

Statistical analysis

Statistical analysis and graphics were performed using R Core Team (2016) statistical software (R: a language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria). Chi-square or Fisher's exact tests were used to examine the associations between clinicopathological parameters (sex, clinical signs, growth pattern, tumor localization, primary site, metastases, and histological differentiation grade). Age and tumor localization were displayed as bar graphs. Average age by tumor localization was displayed as mean ± standard deviation. Age of bovines according to tumor localization was compared using the Student's *t* test when data were normally distributed (determined with the Shapiro–Wilk

test) or the Mann–Whitney U test when not normally distributed. A value of $P < 0.05$ was considered statically significant.

Results

The mean age of affected animals was 7.8 (±2.3) years, ranging from 3 to 13 years. Ninety-six were females and 4 were males. Eighty-nine were crossbreed, 6 Holstein, 4 Jersey and 1 Charolais.

The most common reported clinical signs were weight loss (n=95), ruminal atony (n=77), dysphagia (n=62), coughing (n=59), diarrhea (n=53), regurgitation of ruminal contents through the mouth or nostrils (n=36), bloating (n=35), and salivation (n=30). Less commonly signs were halitosis (n=27), anorexia (n=14), dyspnea (n=8), neck extension (n=6), weakness (n=5) and kyphosis (n=2). Sometimes, the presence of large amounts of undigested food in the feces was reported.

The SCCs were more commonly observed in the oropharyngeal (n=41; Figs 1A and 1B), followed by ruminal (n=35) and esophageal (n=24;) regions (Figs 1C and 1D) of the UDT. Of the 41 oropharyngeal tumors, 26 were located in the pharynx, 10 in base of the tongue and 5 in the epiglottis. Of the 24 esophageal tumors, 9 were in the proximal esophagus, 9 in the middle and 6 in the distal esophagus.

Eighty-one SCCs were of endophytic growth pattern and 19 were exophytic. The endophytic tumors were deeply infiltrating lesions, with relatively few surface manifestations (irregularities in the mucosa with small erosions or ulcers) or extensive ulcers. The exophytic tumors were papillary or verrucous, irregular, ulcerated masses with a broad base or a relatively narrow pedicle. Tumors ranged from 6 to 50 cm in extension, were multilobulated with retention of alimentary content and fetid odor. All SCCs were white-yellowish, firm masses, frequently with yellow spots on cut surface

(corresponding to marked keratinization). Of all endophytic tumors, 13 esophageal tumors had a specific gross presentation as circumferential (annular) masses within the wall with pronounced wrinkling of the mucosa associated with retracted uneven areas and subsequent luminal stenosis (detailed data published elsewhere [18]).

In addition to the SCC of large extension, smaller tumors were observed, sometimes multifocally, along the UDT in 57 cattle. These small tumors were more frequent in the rumen entrance (n = 31) and esophagus (n = 27), followed by epiglottis (n = 17), base of tongue (n = 16), and pharynx (n = 12).

The SCCs were graded as well (n=62), moderately (n=21) or poorly differentiated (n=17). Metastases were present in 52% of cases and these mostly involved regional lymph nodes (retropharyngeal [n=27], gastric [n=13], deep cervical [n=10], mediastinal [n=5], superficial cervical [n=3], submandibular [n=2], mesenteric [n=2] and hepatic [n=2]). Metastasis for liver (n=7), lung (n=5) and spleen (n=4) were less common. Rarely, metastases were observed in greater omentum, urinary bladder, intestine, kidney, trachea and diaphragm.

Throughout the UDT, from the soft palate to the rumen entrance, there were a few or multiple papillomas, ranging from few millimeters in diameter to lesion that were up to 2 cm, pedunculate or sessile, with digitiform projections (cauliflower-like surface), and variable covering keratinization (Fig 2). Microscopically, papillomas were characterized by multiple digitiform projections covered by proliferated squamous epithelium with variable superficial keratinization, supported by central a fibroblastic stalk. Intranuclear viral inclusion bodies were not observed. Three developing phases of papillomas were observed: growing, developing, and a few regressing papillomas. Sometimes, there was carcinomatous transformation of papillomas, grossly characterized by attenuated digitiform projections or ulceration (Fig 3).

Adjacent to the SCCs, the mucosa was often irregular, with focal patches of increased prickle layer or nodules with markedly irregular surfaces, small erosions or ulcers. Histologically, these squamous intraepithelial lesions were characterized by squamous hyperplasia, mild, moderate or severe dysplasia and carcinomas in situ. Moderate to severe dysplasia and carcinoma in situ were frequently accompanied by moderate lymphoplasmacytic inflammatory infiltrate.

Nuclear immunoreactivity for papillomavirus by immunohistochemistry was not observed in any of the (n=93) UDT papillomas. PCR failed to amplify a segment of the BPV genome from the (n=47) extracted samples. Although the BPV-4 PCR sensibility was 1.56x 10^6 DNA copies, the positive control DNA amplified without unspecific reaction. The same was observed in panpapillomavirus PCR (FAP59/64) using bovine papillomavirus type 1 as positive control. Furthermore, the total DNA integrity of all analyzed samples was preserved, and it was confirmed by a PCR using the GAPDH *housekeeping* gene as target (not showed).

A significant ($p<0.001$) association between clinical signs and tumor localization was observed (Table 1). Coughing is the clinical sign associated with oropharyngeal tumors, regurgitation is related to esophageal neoplasms and bloating with ruminal SCCs.

The association between histological differentiation grade and tumor localization was statistically significant ($p=0.007$; Table 2). The well differentiated tumors were more oropharyngeal, and the poorly differentiated tumors were more often observed in esophageal and ruminal regions. There was a trend towards significance ($p=0.063$) association between the variables histological differentiation grade and presence of metastases (Table 2). The poorly differentiated tumors were more associated with the presence of metastases (76,47%).

Oropharyngeal tumors metastasized most frequently (62.5%), followed by ruminal (48.57%) and esophageal (41.67%). Despite this, there was no association between tumor localization (in the three different regions) and the presence of metastasis (p=0.284).

Considering the primary site, epiglottis tumors metastasized most frequently (80% of cases; n=4/5), followed by those located in the distal esophagus (67%; n=4/6), pharynx (61.5%; n=16/26), tongue (50%; n=5/10), rumen entrance (48.5%; n=17/35) and cranial and middle esophagus (33%; n=3/9). However, no association between tumor primary site and presence of metastasis (p=0.486) was observed.

The average age of cattle with oropharyngeal SCCs was 7.39 years old, with statistically significant difference (p=0.018; *t* test) than cattle with esophageal SCCs (8.6 years). No significant difference was found among the mean age of cattle with esophageal and ruminal tumors (7.79 years) or oropharyngeal and ruminal tumors (Fig 4).

No statistical association was observed among other clinical-pathological parameters (sex, growth pattern, primary site of the tumor) analyzed.

Discussion

One hundred cases of SCCs of the UDT of cattle grazing on bracken fern in Southern Brazil were studied. Studies analyzing other aspects of the disease, with 30 [8] and 40 cases [14, 19], out of the 100 studied here, were already published.

Cattle with oropharyngeal SCCs were significantly younger than cattle with esophageal tumors; however, the reason for this was not determined. Cranial neoplasms may progress more rapidly than tumors of other sites in the UDT, or the clinical signs are more rapidly detectable in cranial SCCs [20].

In this study, there was no association between sex and the clinical-pathological parameters evaluated. Although cows are overrepresented in the present study, this does not represent a predisposition for females to develop these tumors. It can be explained by the fact that this category represents the largest bovine population on farms of that geographic area and also because cows have a longer life than the other categories of cattle, being more likely to develop the lesions of chronic poisoning by bracken fern [8]. According to the literature, SCCs associated with chronic bracken fern poisoning takes several years to develop [12, 15]. This is the possible reason the disease was not observed in cattle younger than 3-years-old in this study (mean age of 7.8), considering that many cattle were exposed to and probably consumed the plant since they were born in the affected farms.

Cachexia, ruminal atony and dysphagia were the most common clinical signs in cattle grazing on bracken fern areas, similar to that already observed by other authors [5, 9]. There was a significant association between clinical signs and tumor localization. We observed that coughing is the clinical sign associated with oropharyngeal tumors, regurgitation is related to esophageal tumors and bloating with ruminal neoplasms. Dysphagia was a common clinical sign, regardless of tumor localization. Oropharyngeal SCCs should be highly considered in the differential diagnosis of cattle with coughing grazing on bracken fern infested areas. It is already known that esophagus and stomach deserve particular attention during the examination of animals with regurgitation. The bloating observed in ruminal tumors was the result of a physical defect in eructation of gas produced by normal rumen fermentation (chronic or recurrent secondary bloating) [5]. These physical problems were more commonly caused by obstructions of the esophageal groove by the tumor.

Histological differentiation grade was the main factor predicting the prognosis of SCCs in the UDT of cattle. Poorly differentiated SCCs had the highest rates of metastasis (76,47%; 13/17) when compared to moderately (52,38%) and well (45,16%) differentiated SCCs. Although the prognostic value of histological classification of oral SCCs remains a controversial topic in human medicine and there have been a few studies to determine whether the histological grade also is prognostic in other domestic animals, this should be further evaluated because it is possible that the subtype of the SCCs and histological grade may be useful to predict tumor behavior, especially in cases in which complete clinical staging is not possible [3, 21]. There was also a significant association between histological differentiation grade and tumor localization in the UDT. The well-differentiated tumors were most oropharyngeal, and the poorly differentiated tumors were most in the esophageal and ruminal regions, however, the reason for this was not determined.

The site of the primary tumor is predictive of nodal metastasis and prognosis in human SCCs [22]. In dogs, although neoplasia of the tongue is rare, representing 3–6% of canine oral neoplasia, these neoplasms may have increased metastatic potential, with up to 40% of canine lingual SCCs reported to metastasize to regional lymph nodes compared to metastasis rates 15% for other oral SCCs [3]. Human SCCs also are well recognized to metastasize more quickly from the tongue than from other areas of the oral cavity, probably due to the rich vascular and lymphatic concentration within the tongue and the contraction of tongue muscles promoting the dissemination of neoplastic cells [3, 22]. Comparing with neoplasms that develop at the base of the tongue, rostral SCCs in people have a more favorable prognosis due to more rapid detection, a greater chance of surgical cure, and, because there are fewer lymphatics in the rostral tongue, resulting in less frequent metastases. Similarly, less well-

vascularized areas, such as the glottic larynx, are associated with a lower rate of metastasis [3, 22]. In the present study, tumors on the rostral tongue were not observed. All tumors occurred at the base of the tongue. Metastases were observed in 50% of tongue SCCs. Unlike in dogs, tumors from other localities of the oral cavity metastasized more frequently (61% in the pharynx and 80% in the epiglottis). In spite of this, there was no association between the presence of metastasis and tumor localization in the UDT or tumor primary site in the studies bovines.

SCCs of oral cavity occurred more frequently in the pharynx (63.41%; 26/41), followed by the base of the tongue (24.39%; 10/41) and epiglottis (12.2%; 5/41). In humans, SCCs occur mainly in the oral cavity (67%) followed by pharyngeal region (30%) [1], it is suggested that higher exposure to tobacco smoking and alcohol drinking are required to induce oropharyngeal than oral cancer [23]. It is important to point out that the main toxic principle of bracken fern, a norsesquiterpene ptaquiloside, unfolds into a reactive dienone (carcinogenic and mutagenic) in places of alkaline pH, as under influence of saliva [24, 25].

Metastases were present at a much higher frequency (52% of cases) in our study when compared to oral SCCs in dogs and cats (around 15% of nodal metastasis) [3, 20]. Regional lymph node metastasis is most often found with poorly differentiated SCCs or neoplasms that have been present for a long time before they are diagnosed or excised [16]. Poorly differentiated SCCs had the highest rates of metastasis in this study. Additionally, the SCCs in cattle are usually diagnosed late, when the animal shows evident clinical signs due to large or critically located (i.e. in the rumen entrance) neoplastic masses. Therefore, these two factors may have contributed to the high frequency of metastases in the present study.

Endophytic SCCs were much more frequent (81% of cases) than exophytic tumors, in accordance with the local invasive nature of this tumor, but no statistical association was observed between the growth pattern and clinical or pathological factors. Of all endophytic tumors, 13 had a specific gross presentation as annular stenotic esophageal SCCs, which were detailed elsewhere [18]. In humans, head and neck endophytic SCCs have worst prognosis than exophytic tumors [26-29]. Nevertheless, other studies have established that thickness of the tumor is the most reliable predictive factor that had significant value for subclinical nodal metastasis, local recurrence, and survival in patients with oral cancer [30-32]. The tumor thickness is measured from the deepest tumor invasion to the presumed original surface level, that is, ignoring exophytic growth or assessing the original surface level in ulcerated tumors [2, 30]. Additionally, the higher incidence of subclinical nodal metastasis and poor prognosis found in endophytic and expansive tumors in some studies can be probably caused by their association with thicker tumors in humans [32]. More studies are needed to determine if tumor thickness also influences the prognosis of SCCs in cattle.

The presence of BPV DNA was investigated in papillomas by PCR with primers specific for BPV-4 and with panpapillomavirus degenerate primer. Despite the relatively low sensitivity of the PCR used in this study, high copy numbers of BPV-4 DNA sequences have been regularly detected in both naturally occurring or experimentally induced papillomas, as observed by Campo et al. [13] who detected BPV-4 DNA in 61 out of 67 papillomas in which the copy number could be as high as 1×10^5 genomes per cell, indicating the presence of high viral load in infected tissues.

The presence of papillomavirus antigens was investigated by immunohistochemistry using a monoclonal antibody cocktail against HPV. In the

absence of a commercially available specific primary antibody for BPV-4, a broad spectrum anti-HPV antibody was used, similar (against the same HPV subtypes) to the ones that have been successfully used to identify papillomavirus infection in cattle [33], horse [34], and ferret [35]. It was found only one illustration in a review article showing the use of anti-HPV antibodies (not specified) revealing the presence of papillomaviral L1 protein within the superficial epithelium, confirming the presence of papillomaviral replication, in an oropharyngeal papilloma in a cow [36].

The absence of DNA detection of BPV-4 by PCR, the absence of nuclear immunoreactivity for papillomavirus by immunohistochemistry and the absence of intranuclear inclusion bodies in papillomas suggest that papillomas of the UDT in cattle may not be necessarily associated with papillomavirus infection in Southern Brazil.

Additionally, the presence of squamous intraepithelial lesions found throughout the UDT epithelium that do not appear to pass through a papillomatous stage may demonstrate direct action of bracken fern carcinogens in the epithelium without the need for viral involvement [37]. In Brazil, both CCEs and papillomatosis of the UDT occur only in areas with presence of bracken fern [15].

Oral papillomatosis in cattle is rare and it is observed in specific cases of immunosuppression [38]. According to the papilloma-carcinoma theory proposed by Campo et al. [12], persistence of alimentary papillomatosis, with progression to SCCs, is induced by immunosuppression due to lymphopenia caused by *Pteridium* spp. toxicity. In a study of spontaneous cases of bracken fern-related SCCs of the UDT in Southern Brazil, lymphopenia was observed only in three out of 40 cases [19].

The absence of papillomavirus DNA or antigen in the papillomas, the absence of lymphopenia in most cows and the presence of squamous intraepithelial lesions in the UDT, keep open the possibility that papillomas of the UDT may not be necessarily

associated with papillomavirus in cattle from our region and that papillomas and SCCs could be both independently induced by bracken fern. This possibility was also considered in a broad bovine and human comparative papillomavirus review [36]. Consequently, BPV-4 infection apparently is not a cofactor involved in carcinogenesis of alimentary SCCs in cattle from Southern Brazil. All these findings point out to the need for further investigations of the etiopathogenesis of this disease using other molecular and antigenic tests, such as next generation sequencing and BPV-4 specific monoclonal antibodies.

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Competing Interests

The authors have declared that no competing interests exist.

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Figures legends

Fig 1. Squamous cell carcinoma of the upper digestive tract of cattle associated with chronic poisoning by bracken fern located in the base of the tongue (A), pharynx (B), distal esophagus (C) and rumen (D).

Fig 2. Oropharyngeal papillomas associated with chronic poisoning by bracken fern on a cow.

Fig 3. Ruminal transforming papillomas associated with chronic poisoning by bracken fern in a cow.

Fig 4. Squamous cell carcinomas (SCCs) of the upper digestive tract (UDT) in cattle associated with chronic poisoning by bracken fern. The average age of cattle according to tumor localization in the UDT. Cattle with oropharyngeal SCCs (7.39 years old) were younger than those with esophageal SCCs (8.6 years, *p=0.018). Although cattle with oropharyngeal SCCs were, on average, younger than those with ruminal SCCs (7.79 years), the difference was not statistically significant.

559 **Table 1. Association between clinical signs and SCCs localization in the upper**
560 **digestive tract in cattle grazing on bracken fern areas**

	Tumor localization		
	Oropharyngeal (n=41)	**Esophageal (n=24)**	**Ruminal (n=35)**
Clinical signs*			
Weight loss: n=95	41 (43,16%)	22 (23,16%)	32 (33,68%)
Ruminal atony: n=77	32 (41,56%)	17 (22,08%)	28 (36,36%)
Dysphagia: n=62	26 (41,93%)	16 (25,81%)	20 (32,26%)
Coughing: n=59	32 (54,24%)	12 (20,34%)	15 (25,42%)
Diarrhea: n=53	16 (30,19%)	14 (26,41%)	23 (43,40%)
Regurgitation: n=36	8 (22,22%)	17 (47,22%)	11 (30,56%)
Bloating: n=35	3 (8,57%)	10 (28,57%)	22 (62,86%)
Salivation: n=30	14 (46,66%)	8 (26,67%)	8 (26,67%)

561 *Significant ($p<0.001$) association by Chi-square or Fisher's exact tests between tumor
562 localization and clinical signs.

Table 2. Association between histological differentiation grade and tumor localization or presence of metastasis of SCCs of the upper digestive tract in cattle grazing in bracken fern areas

	Histological differentiation grade		
	WD (n=62)	**MD (n=21)**	**PD (n=17)**
Tumor localization[a]			
Oropharyngeal: n=41	32	8	1
Esophageal: n=24	11	6	7
Ruminal: n=35	19	7	9
Metastases[b]			
Presence: n=52	28	11	13
Absence: n=48	34	10	4

WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; [a]Significant (p=0.007) association by Chi-square or Fisher's exact tests between histological differentiation grade and tumor localization; [b]Trend towards significance (p=0.063) association by Chi-square or Fisher's exact tests between the variables histological differentiation grade and presence of metastases.

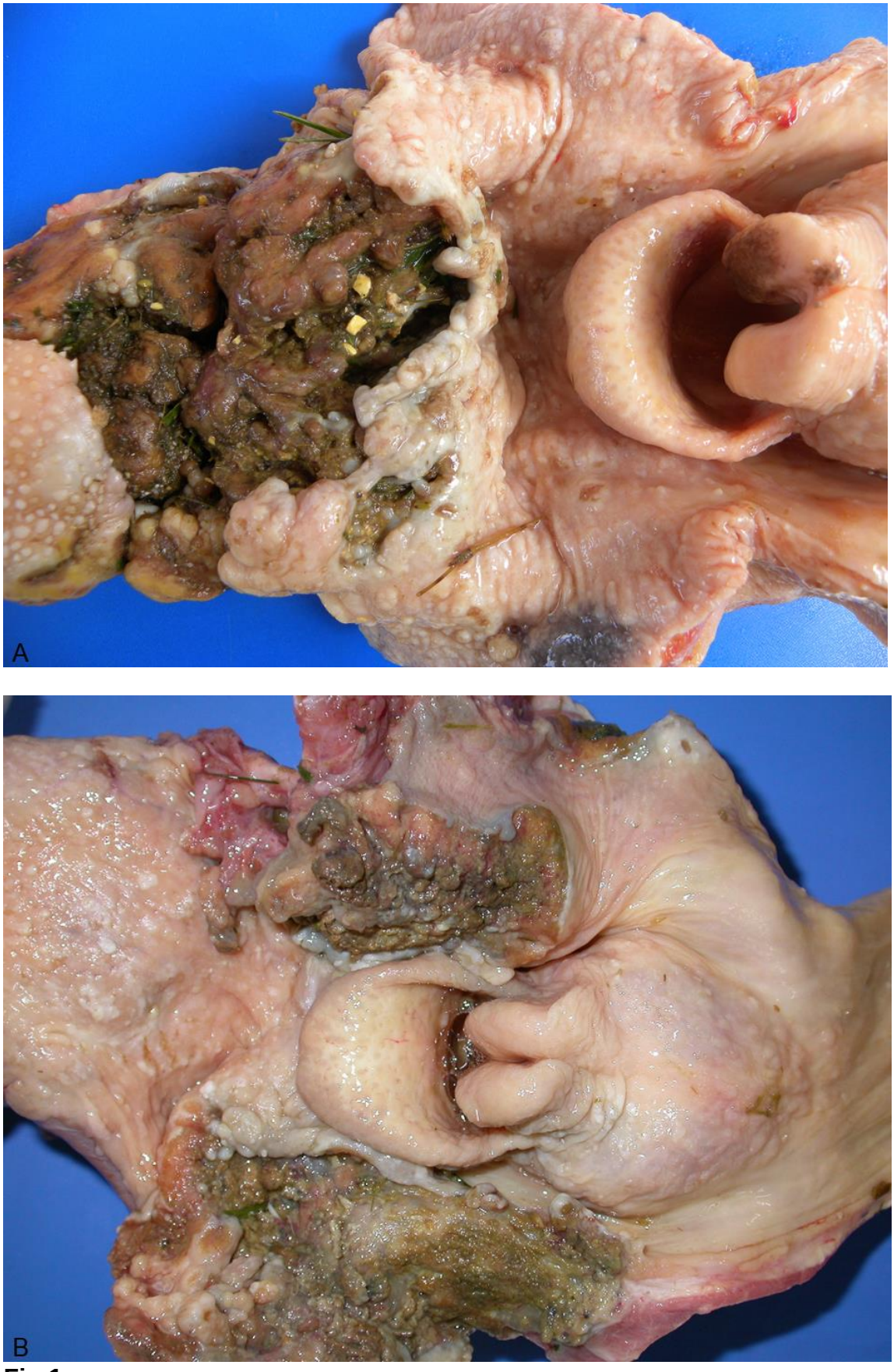
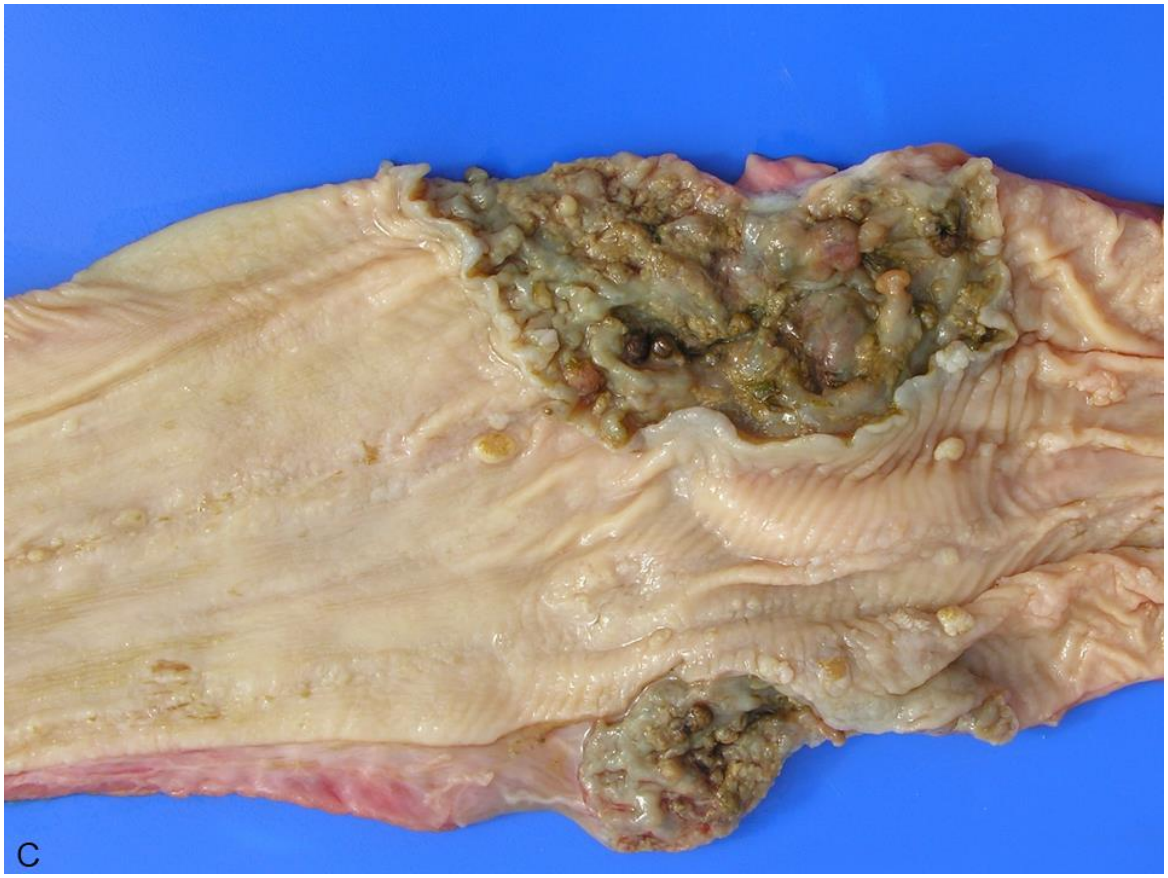


Fig 1



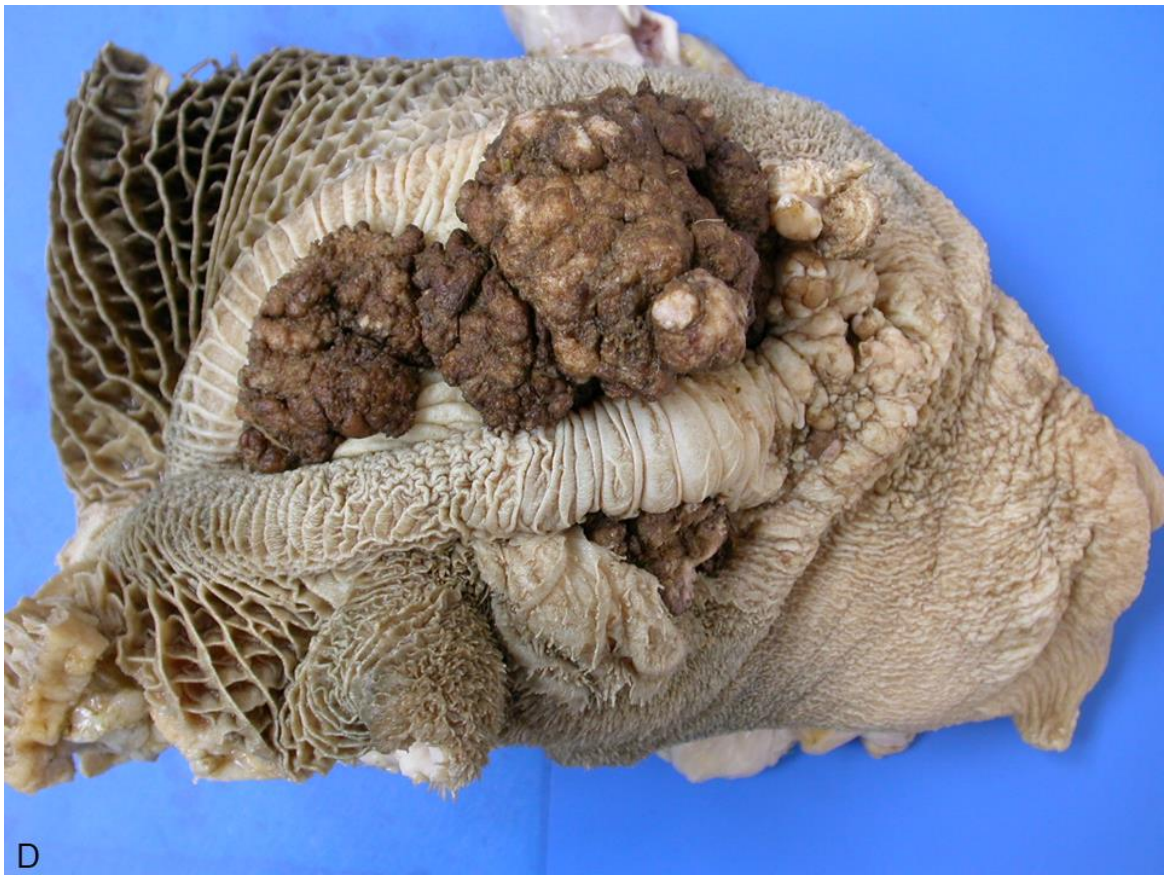
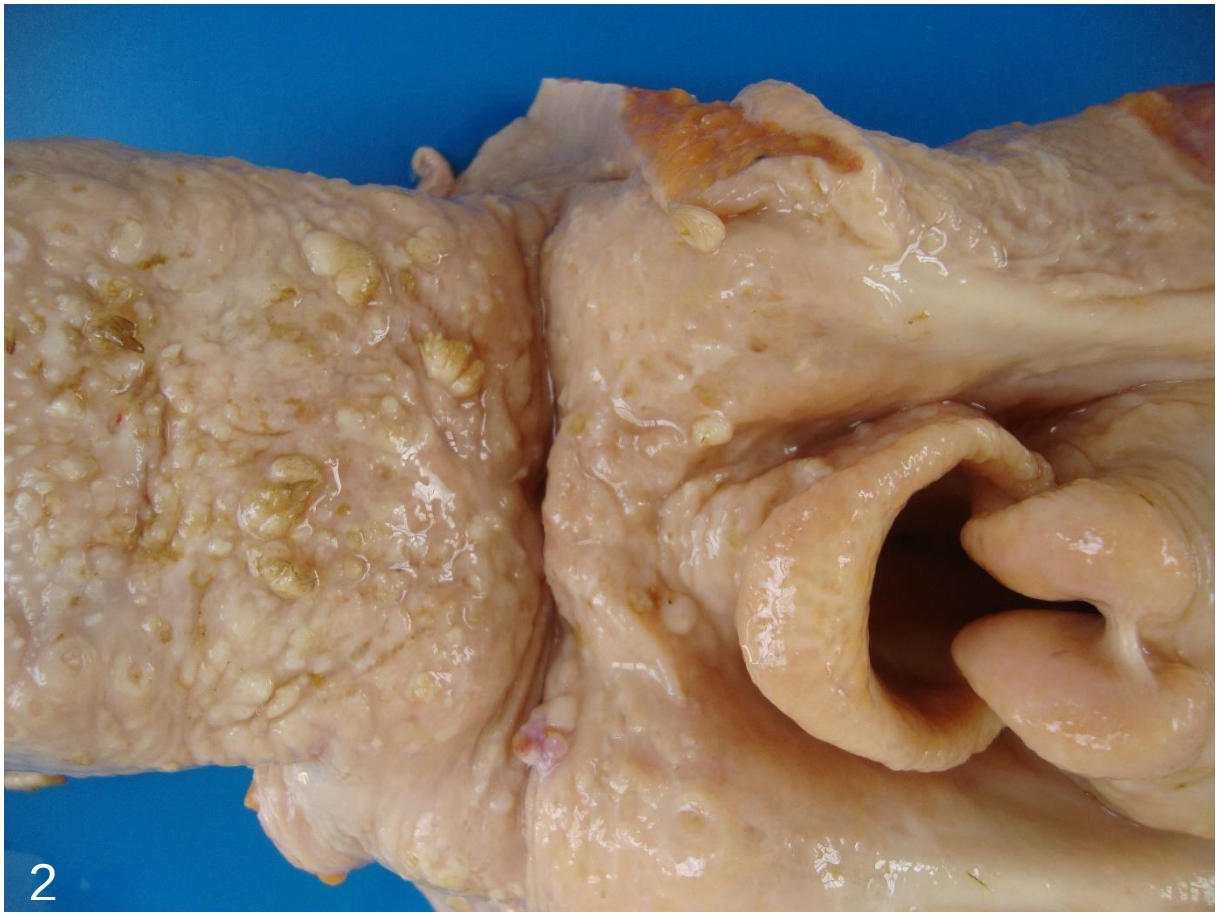


Fig 1



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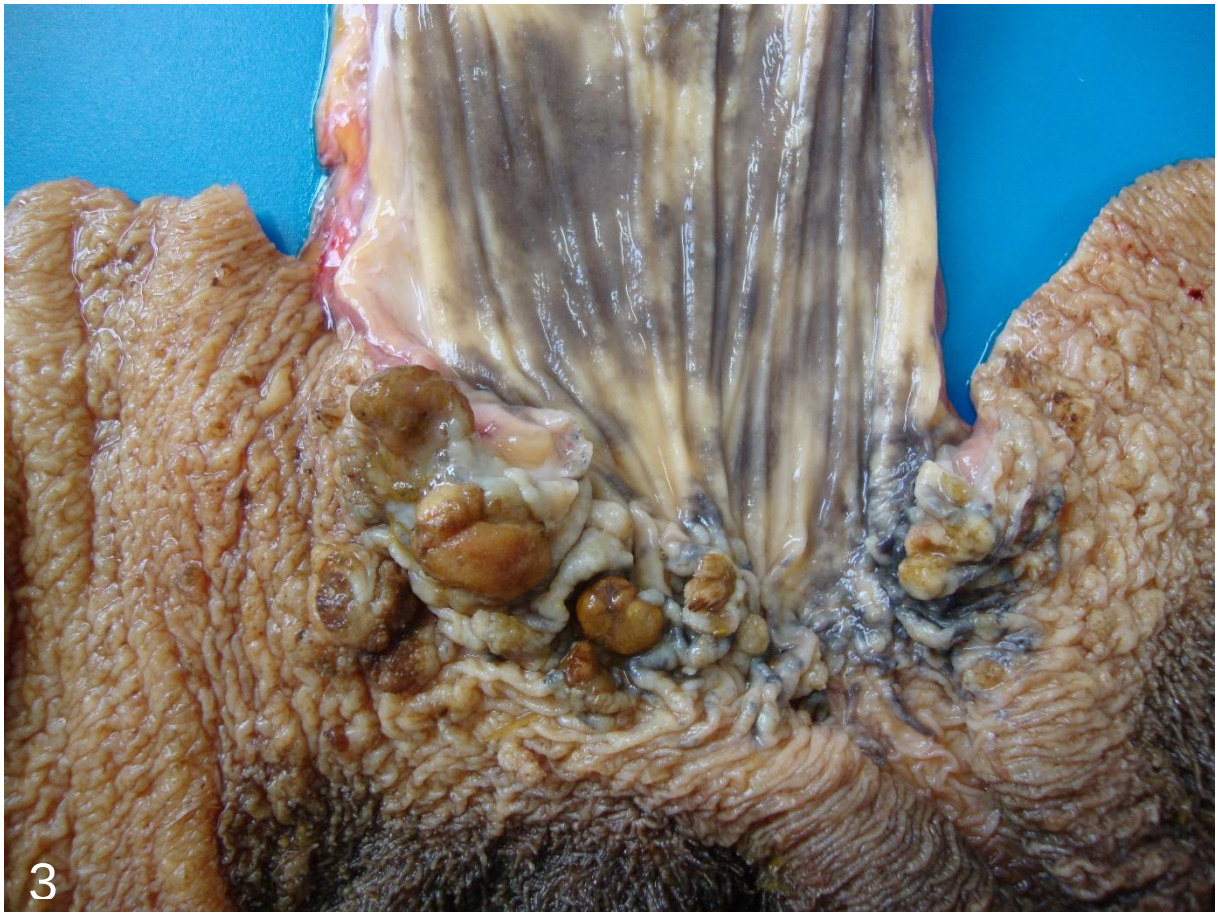

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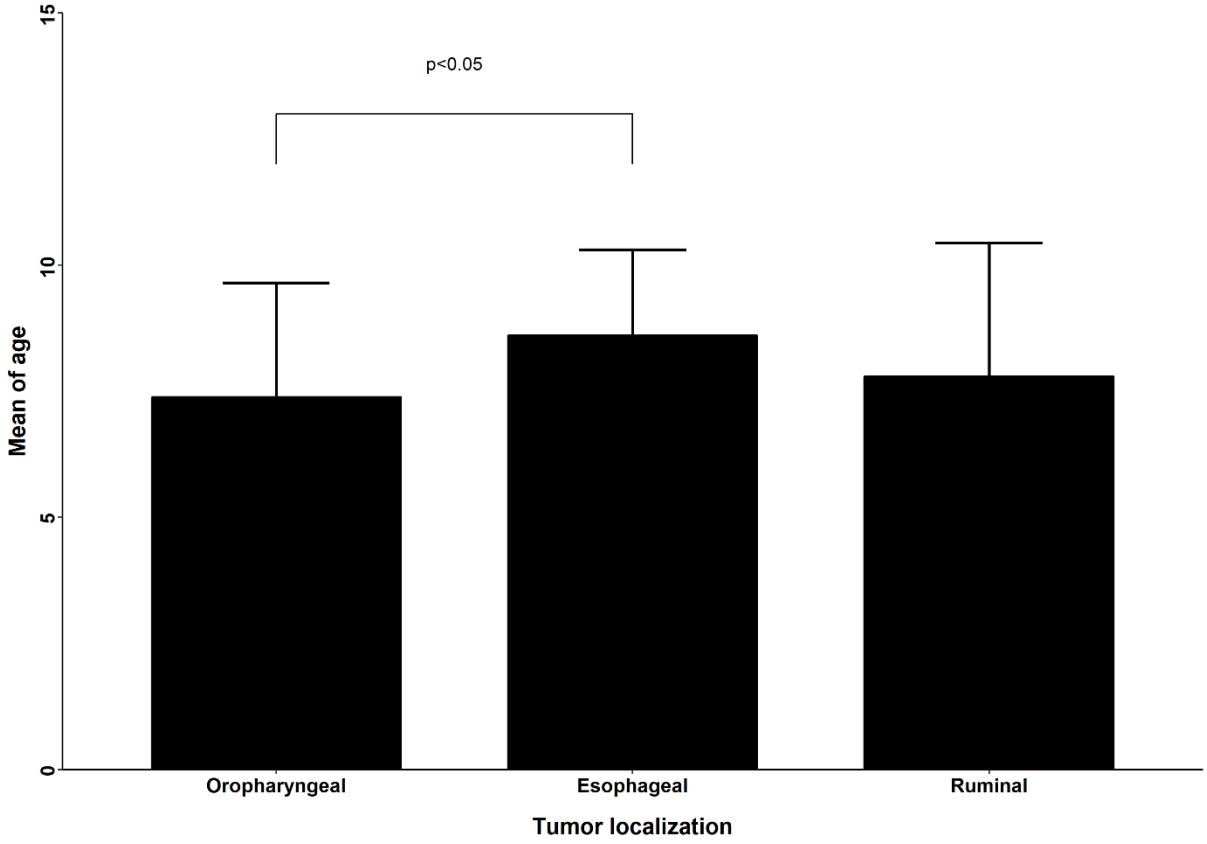
p<0.05
Mean of age
0
5
10
15
Oropharyngeal
Esophageal
Ruminal
Tumor localization

581

582 **Fig. 4**

4 ARTIGO 2 - ANNULAR STENOTIC OESOPHAGEAL SQUAMOUS CELL CARCINOMA IN CATTLE EXPOSED NATURALLY TO BRACKEN FERN (*Pteridium arachnoideum*)

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SPONTANEOUSLY ARISING DISEASE

Short Title: Bracken Fern-associated Carcinoma in Cattle

Annular Stenotic Oesophageal Squamous Cell Carcinoma in Cattle Exposed Naturally to Bracken Fern (*Pteridium arachnoideum*)

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Summary

Oesophageal squamous cell carcinomas (SCCs) may be observed as exophytic masses or ulcerative or infiltrative endophytic neoplasms. However, in cattle, there is also an uncommon gross presentation as an annular stenotic thickening of the oesophageal wall. Thirteen cases of annular stenotic oesophageal SCCs in cattle grazing in bracken fern (*Pteridium arachnoideum*) areas are reported. The lesions consisted of endophytic masses, focally extensive, firm and circumferential (annular) in the oesophageal wall. Pronounced wrinkling of the mucosa, with retracted uneven areas and subsequent luminal narrowing (stenosis), was observed in all cases. Papillomas and squamous intraepithelial lesions also were observed in these cases. The SCCs were graded as well differentiated (n = 7), moderately differentiated (n = 5) or poorly differentiated (n = 1). The neoplastic keratinocytes were surrounded by moderate to abundant fibrous connective tissue (a desmoplastic reaction), that was better demonstrated by Masson's trichrome stain. Picrosirius red-stained sections showed abundant collagen type I fibres, which contributed to the stenosing characteristics of this tumour. Although it might be easily misdiagnosed as oesophageal scar tissue, the oesophageal SCCs of cattle grazing bracken fern may have a distinctive gross appearance that should be included in the differential diagnosis of oesophageal stenosis.

Keywords: bracken fern; cattle; oesophageal squamous cell carcinoma; oesophageal stenosis

Introduction

Oesophageal cancer is the ninth most common cancer in man and the sixth most common cause of death from cancer (Fitzmaurice *et al.*, 2015). In domestic animals, primary tumours of the oesophagus are rare (Head *et al.*, 2002). Malignant neoplasms of the oesophagus in

ruminants are typically extremely rare (Uzal *et al.*, 2016); however, in several countries, squamous cell carcinomas (SCCs) of the upper digestive tract (UDT) of cattle are relatively common and they are associated with papillomas and chronic ingestion of bracken fern (*Pteridium* spp.) (Tokarnia *et al.*, 1969; Jarrett *et al.*, 1978; Souto *et al.*, 2006; Uzal *et al.*, 2016). In Brazil, two species of *Pteridium* are described, *Pteridium caudatum* and *Pteridium arachnoideum* (Prado and Sylvestre, 2010). These species were previously referred to as varieties of *Pteridium aquilinum* (Thomson, 2000). In the state of Rio Grande do Sul, Brazil, only *P. arachnoideum* is described (Prado and Sylvestre, 2010).

Oesophageal SCCs may be observed as exophytic masses or ulcerative or infiltrative endophytic neoplasms (Gabbert *et al.*, 2000). However, there is also an uncommon gross presentation as an annular stenotic thickening of the oesophageal wall. This presentation of oesophageal SCCs has been mentioned since the first reports of the disease in cattle (Tokarnia *et al.*, 1969; Head *et al.*, 2002; Souto *et al.*, 2006; Lucena *et al.*, 2011; Masuda *et al.*, 2011), without further studies about its particular clinical and pathological aspects. According to the reviewed literature, this unique presentation of oesophageal SCC has not been reported in other domestic animals (Head *et al.*, 2002) or in man (Gabbert *et al.*, 2000). At necropsy examination, these annular stenotic tumours may be misinterpreted as simple oesophageal stenotic scars by field veterinarians who are unaware of this morphological presentation.

The aim of this study was to elucidate the clinical and pathological aspects of annular stenotic oesophageal SCC in cattle grazing in bracken fern areas. The pathological aspects of this unique neoplasm and associated lesions were compared with human SCC.

Material and Methods

Thirteen cases of annular stenotic oesophageal SCC were selected from a total of 100 cattle with SCC in the UDT (41 oral, 24 oesophageal and 35 ruminal tumours) received between

September 2003 and August 2014. Samples were obtained from necropsy examinations performed by veterinary pathologists in our laboratory or by a trained field veterinarian, who sent the whole UDT (from the tongue to the rumen) to our laboratory in all suspected cases of chronic poisoning by bracken fern. These cattle had grazed for years on pastures with high levels of contamination with bracken fern (*P. arachnoideum*). The farms were all within the Central region of the State of Rio Grande do Sul, southern Brazil. The age, sex, and clinical signs of cattle and locations of the tumour in the oesophagus (upper [proximal], middle and lower [distal] third) were recorded. The clinical signs were identified by clinical examinations performed by the same trained field veterinarian.

Samples of the lesions were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections were stained with haematoxylin and eosin (HE). Microscopically, each SCC was graded as well, moderate or poorly differentiated, according to the criteria of Head *et al.* (2002). Serial sections were also stained with Masson's trichrome for quantification of the collagen present within desmoplastic areas. Additionally, picrosirius red staining was applied and the sections were analysed under polarized light in order to differentiate collagen types I (yellow, orange or red fibres) and III (green fibres) (Whittaker *et al.*, 1994). Immunohistochemistry (IHC) was employed with the streptavidin–biotin–peroxidase method using a rabbit polyclonal anti-bovine pan-cytokeratin antibody (Dako, Carpinteria, California, USA; 1 in 2,000 dilution) for evaluation of the morphological patterns of neoplastic cell migration and invasion (Masuda *et al.*, 2011).

Results

The age, sex, clinical signs of cattle, locations of the tumour in the oesophagus and specific features of each tumour are summarized in Table 1. The lesions were grossly similar in all cases. They were white endophytic masses, focally extensive, firm and circumferential

(annular) within the oesophageal wall. In the affected areas, there was pronounced wrinkling of the mucosa, with retracted uneven areas and subsequent luminal narrowing (stenosis), as observed in Fig. 1a.

Adjacent to the SCCs, the oesophageal mucosa was often irregular with many small erosions or ulcers and slightly elevated plaques, or nodules with markedly irregular surfaces (Fig. 1b). These lesions were white, often with small multifocal yellow spots. Throughout the UDT, from the soft palate to the rumen entrance, there were multiple papillomas ranging from 1 mm to 1 cm in diameter.

Microscopically, the SCCs were characterized by proliferation of well- to poorly-differentiated neoplastic keratinocytes arranged in islands, ribbons, cords, small aggregates or individual cells (Fig. 1c). The islands often had a necrotic centre or contained concentric lamellae of keratin ('keratin pearls'). When there was necrosis of the neoplastic islands, keratin was mineralized and surrounded by connective tissue. Some individual cells showed an increase in cytoplasmic eosinophilia (individual keratinization). Neoplastic keratinocytes were round to polyhedral, with abundant cytoplasm that varied from pale to brightly eosinophilic. Nuclei were vesicular, round to oval, with single, central, prominent nucleoli. Nuclear and cellular pleomorphism ranged from mild to severe. The stenotic oesophageal SCCs were graded as well (n = 7), moderate (n = 5) or poorly differentiated (n = 1).

The neoplastic keratinocytes were surrounded by moderate to abundant fibrous connective tissue (desmoplastic reaction). In all cases, they invaded the muscular layer and eventually also infiltrated the tunica adventitia (in the cervical region) or the serosa (in the thoracic and abdominal regions). The mucosa was less affected in all cases. The fibrous tissue was blue with Masson's trichrome staining (Fig. 1d) and varied from moderate to abundant in all degrees of differentiation. Picrosirius red-stained sections, under polarized light, showed thick orange to red fibres, compatible with type I collagen (Fig. 1e). The morphological

patterns of cell migration and invasion by neoplastic keratinocytes, as described above for each histological grade, were best characterized using IHC for cytokeratin. Islands, ribbons, and cords were observed more in well-differentiated SCCs, while cords, small aggregates or individual cells were more frequent in the less differentiated ones (Fig. 1f).

There were also squamous intraepithelial lesions (SILs), characterized by squamous hyperplasia, moderate or severe dysplasia and carcinomas in situ of the oesophageal epithelium, adjacent to the stenotic SCCs. Multiple small epithelial erosions and ulcerations were observed throughout the oesophagus.

Discussion

In domestic animals, oesophageal stenosis may result from rare intramural or intraluminal neoplasia or, commonly, by external compression (i.e. enlarged hyperplastic or neoplastic thyroids, or neoplasia of the thymus or cervical/mediastinal lymph nodes) (Uzal *et al.*, 2016). Although it might be easily misdiagnosed as oesophageal scar tissue, the oesophageal SCCs of cattle grazing *P. arachnoideum* in this study may have a distinctive gross appearance that should be included in the differential diagnosis of oesophageal stenosis.

Clinical signs observed in this study were similar to those observed in man, in which the most common symptoms of advanced oesophageal cancer are dysphagia, weight loss, retrosternal or epigastric pain and regurgitation (Gabbert *et al.*, 2000). The macroscopic classification of advanced oesophageal SCC in man involves three major patterns: fungating, ulcerative and infiltrating. The fungating pattern is characterized by predominantly exophytic growth, while in the ulcerative pattern the tumour growth is predominantly intramural, with central ulceration and elevated ulcer edges. The infiltrative pattern, which is least common, also shows a predominantly intramural growth, but causes only a small mucosal defect (Gabbert *et al.*, 2000). The annular stenotic pattern of oesophageal SCCs in cattle of the

present study is similar to the infiltrative pattern in man, but in cattle, all layers of the oesophagus are affected.

The oesophageal SCCs reported in this study were characterized by a fibrous desmoplastic reaction; this change was not found in human oesophageal SCC (Gabbert *et al.*, 2000). For reasons that are unclear, the amount of stroma produced by different neoplasms varies considerably among tumour types and animal species. Certain carcinomas, such as gastric, transitional cell and mammary carcinomas, are more prone to develop desmoplasia than other neoplasms. These masses are firm and rubbery and the stroma can comprise a larger proportion of the mass than the tumour cells (Cullen *et al.*, 2002). The degree of desmoplasia in oesophageal SCCs in man is variable (Gabbert *et al.*, 2000) and published data concerning the amount of desmoplastic reaction in oesophageal cancers of domestic animals was not found. Growth factors and cell mediators released by fibroblasts or neoplastic cells could explain the abundance of the desmoplastic tissue, which could influence cancer progression and tumour cell mobility (Cullen *et al.*, 2002).

The results of the present study demonstrate that collagen fibres observed in the desmoplastic reaction are compatible with type I collagen. Pancreatic ductal adenocarcinoma in man is also characterized by pronounced tumour-associated desmoplasia, primarily composed of type I collagen (Armstrong *et al.*, 2004; Shields *et al.*, 2011). Although it is well known that type I collagen functions as a barrier to invasion, analysis of these tumours in people has shown that increased collagen expression can also be associated with poor prognosis and with increased metastasis (Shields *et al.*, 2011). Type I collagen was associated with proliferation of cancer cells and reduced apoptosis in pancreatic cancer (Armstrong *et al.*, 2004). These experiments elucidated a mechanism by which the desmoplastic reaction in pancreatic cancer may form and, via the collagen within it, promote the malignant phenotype of pancreatic cancer cells, resulting in significant detriment to the

host (Armstrong *et al.*, 2004). Type I collagen appears to contribute to the stenosing characteristics of the oesophageal SCCs reviewed in this study. Further studies are necessary to identify whether type I collagen observed in annular stenotic oesophageal SCCs of cattle grazing in bracken fern areas is correlated with poor prognosis.

In the human literature, the incidence of oesophageal SCC is high in specific ethnic groups and certain geographical locations (i.e. Eastern and Southern Africa and in Eastern Asia) (Ferlay *et al.*, 2010; Jemal *et al.*, 2011) and is also influenced by environmental factors (e.g. tobacco use and excessive alcohol consumption) and genetic factors (e.g. mutations in enzymes that metabolize alcohol) (Gabbert *et al.*, 2000; Engel *et al.*, 2003; Yang *et al.*, 2007). Some human populations also eat young bracken fern shoots and a few epidemiological studies have shown a close association between bracken fern consumption and cancers of the upper alimentary tract (Alonso-Amelot and Avendano, 2001, 2002). In cattle, chemical agents are also involved in the high incidence of oesophageal SCC. One of the chemical compounds of bracken fern is ptaquiloside, a highly mutagenic and carcinogenic factor (Fenwick, 1988). In addition, quercetin also has been implicated as a carcinogen present in bracken fern (Pamukcu *et al.*, 1980). In cattle, it is unknown whether genetic factors, such as mutations in enzymes, may also contribute to the occurrence of oesophageal (or other UDT) tumours. Carcinogen and papillomavirus interactions have also been implicated in the pathogenesis of the disease in both cattle (Jarret *et al.*, 1978; Campo *et al.*, 1994) and man (Petrick *et al.*, 2014).

Alimentary papillomatosis occurs in association with upper alimentary SCCs in cattle grazing on bracken fern-containing pastures (Tokarnia *et al.*, 1969; Jarrett *et al.*, 1978; Souto *et al.*, 2006; Lucena *et al.*, 2011) and this was also observed in the cattle of the present study. According to pathogenesis proposed by Campo *et al.* (1994), papillomas would serve as an expanded target, due to the constant cell replication induced by bovine papillomavirus type-4

(BPV-4), for bracken fern chemical carcinogens to act synergistically with the virus in the development of SCCs. According to a papillomavirus review (Munday, 2014), such observations suggest a role of BPV-4 in the development of upper alimentary SCCs. However, the requirement of *Pteridium* spp. for neoplasia makes it hard to determine the precise role of BPV-4 in cancer development. BPV-4 appears likely to be an important cofactor in carcinogenesis, but it cannot be excluded that papillomas and SCCs are both independently induced by bracken fern (Munday, 2014).

In man, there is also controversy as to whether human papillomavirus (HPV) infection is associated with the development of oesophageal SCCs (Ohashi *et al.*, 2015). HPV infection is associated with tumorigenesis in cervical cancer, as well as in head and neck SCC (Adams *et al.*, 2014). However, a relationship between HPV infection and development of SCCs in the oesophagus has not been observed consistently (Gabbert *et al.*, 2000). Although there have been many HPV and oesophageal cancer studies, the International Agency for Cancer Research (IACR) has concluded that the findings have been very inconsistent geographically. The extreme variations in HPV detection in these studies may be related to the lack of standardized testing methods. There are areas, particularly in Asia, where HPV is more commonly detected in oesophageal cancer (IARC, 2012). A comprehensive genetic analysis of several viruses including HPV, hepatitis B virus and human herpesvirus found no viral integration into the genome of oesophageal SCCs (Song *et al.*, 2014) or in laryngeal SILs (Gale *et al.*, 2009).

Hyperplastic and dysplastic SILs and carcinomas in situ in the oesophagus were also observed in the present study. In man, laryngeal and oesophageal SILs are caused by the toxic principles of smoking and alcohol abuse (Gabbert *et al.*, 2000; Gale *et al.*, 2009). SILs have already been described in cattle grazing in bracken fern-containing pastures (Tokarnia *et al.*, 1969; Souto *et al.*, 2006) and were studied in detail more recently (Masuda, 2010). The

presence of these epithelial lesions may support the possibility of a direct role of the toxic principles of *Pteridium* spp. in tumorigenesis without the requirement for a previous papillomatous lesion (Masuda, 2010).

In summary, the annular stenotic presentation is a distinctive type of oesophageal SCC in cattle grazing in bracken fern-contaminated pastures. Type I collagen fibres contribute to the stenosing characteristics of this oesophageal tumour. This tumour presentation must be considered in the differential diagnosis of oesophageal stenosis of cattle.

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Conflict of Interest Statement

The authors declare no conflict of interest with respect to the publication of this paper.

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Figure Legends

Fig. 1. Oesophageal SCC. (a) Note the annular stenotic SCC within the proximal oesophagus showing marked wrinkling of the mucosa. Bar, 1 cm. (b) Adjacent to a SCC (not shown), there are multifocal small erosions or ulcers (arrowheads) and slightly elevated plaques or nodules (arrows) with markedly irregular mucosal surface, corresponding histologically to squamous intraepithelial lesions. Bar, 1.5 cm. (c) Well-differentiated SCC. Islands (with central keratinization) and anastomosing ribbons and cords of neoplastic keratinocytes are surrounded by an inflammatory infiltrate and desmoplastic reaction. HE. Bar, 50 µm. (d) Well-differentiated SCC. Neoplastic islands of keratinocytes are surrounded by abundant collagen fibres (in blue) of the desmoplastic reaction. Masson's trichrome stain. Bar, 50 µm. (e) Thick orange to red fibres, compatible with type I collagen, surrounding neoplastic islands. Picrosirius red stain, under polarized light. Bar, 50 µm. (f) Small aggregates of keratinocytes typical of a poorly-differentiated SCC. IHC. Bar, 50 µm.

Table 1
Epidemiological, clinical, and pathological aspects of annular stenotic oesophageal SCCs in cattle exposed naturally to bracken fern

Case	*Sex*	*Age (years)*	*Clinical signs*	*Tumour localization in the oesophagus*	*Features of the tumour*
1	F	9	Ruminal atony, bloating, diarrhoea, weight loss	Proximal	8 cm, non-ulcerated
2	F	8	Cough, regurgitation, ruminal atony, bloating, weight loss	Proximal	6.5 cm, with small, focal erosion
3	F	9	Dysphagia, regurgitation, ruminal atony, weight loss	Middle	12 cm, with small, multifocal ulcers
4	F	8	Salivation, dysphagia, cough, regurgitation, weight loss	Middle	7 cm, with small, multifocal erosions
5	F	10	Dysphagia, cough, regurgitation, ruminal atony, bloating, diarrhoea, weight loss	Middle	7 cm, with focal ulcer and multifocal punctate keratin deposits on cut surface
6	F	8	Dysphagia, regurgitation, weight loss	Middle	With focal ulcer
7	F	12	Salivation, dysphagia, ruminal atony, bloating, diarrhoea, weight loss	Distal	11.5 cm
8	F	7	Dysphagia, cough, regurgitation, ruminal atony, bloating, diarrhoea, weight loss	Proximal	10 cm, with focal ulcer with food impaction
9	F	7	Salivation, dysphagia, ruminal atony, bloating, diarrhoea, weight loss	Proximal	12 cm, with small, multifocal ulcers; multifocal necrosis and punctate keratin deposits on cut surface
10	F	10	Dysphagia, regurgitation, ruminal atony, diarrhoea, weight loss	Proximal	13.5 cm, with focally extensive ulcer
11	F	8	Salivation, dysphagia, cough, regurgitation, ruminal atony, diarrhoea	Proximal	Non-ulcerated
12	F	Unknown	Salivation, cough, regurgitation, ruminal atony, diarrhoea, weight loss	Middle	10 cm
13	F	4	Salivation, weight loss	Proximal	With focally extensive ulcer

F, female.

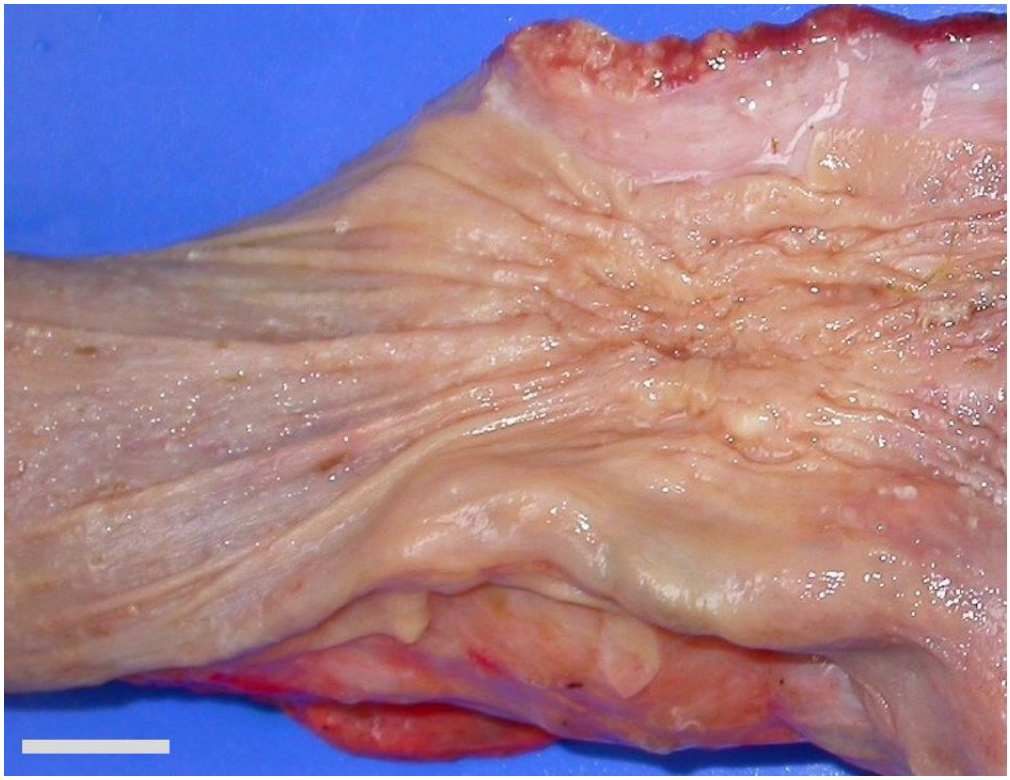

Fig. 1 (a)

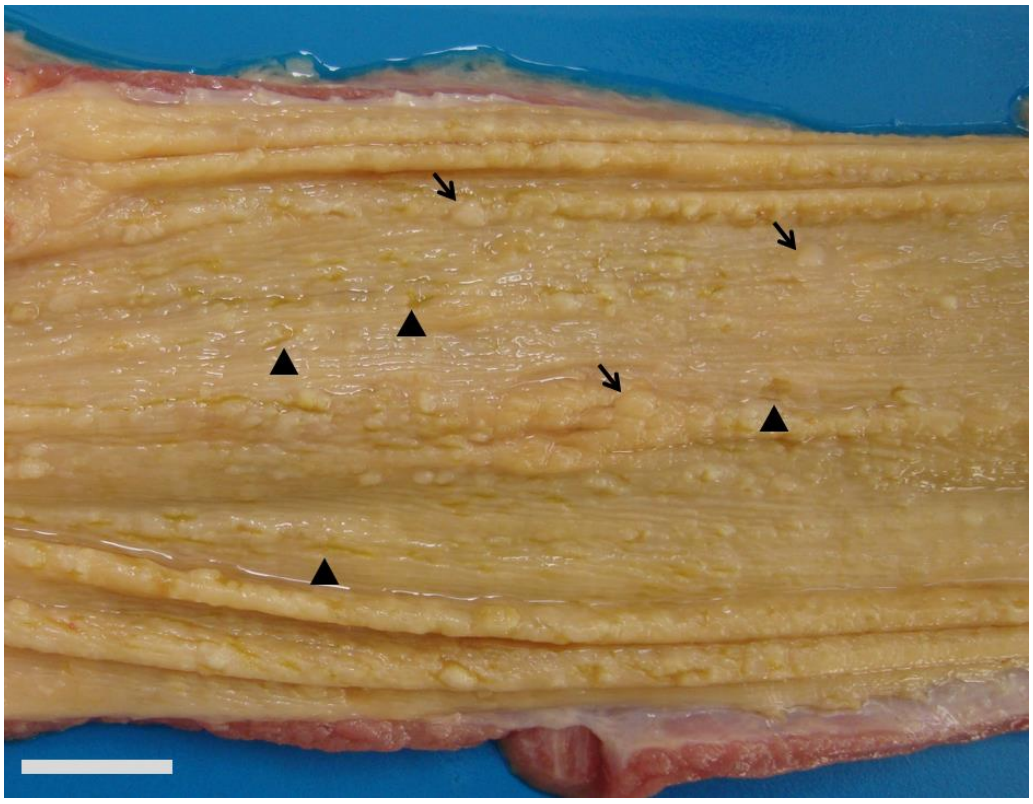

Fig. 1 (b)

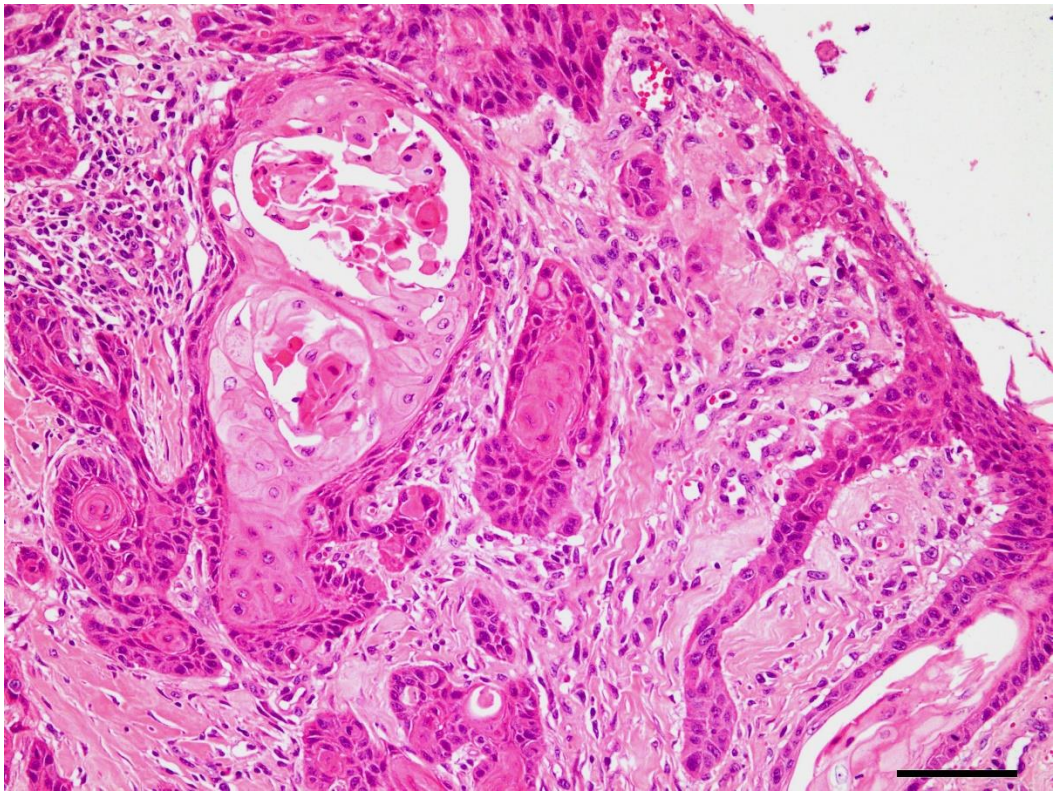

Fig. 1 (c)

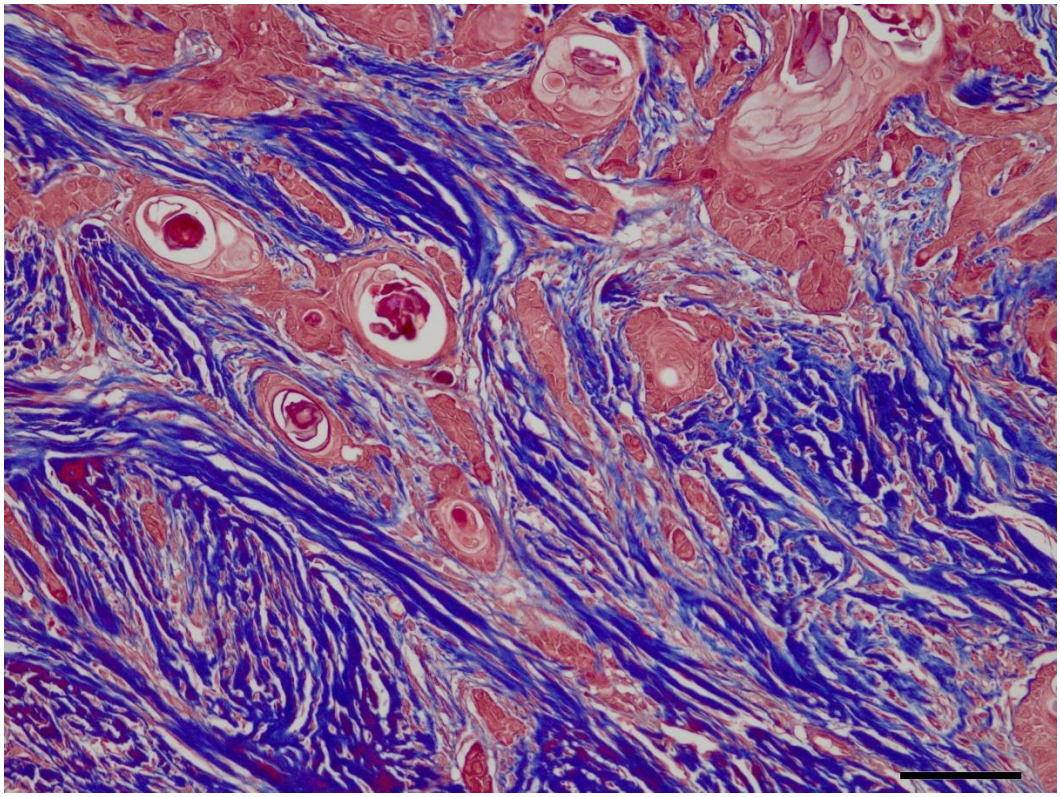

Fig. 1 (d)

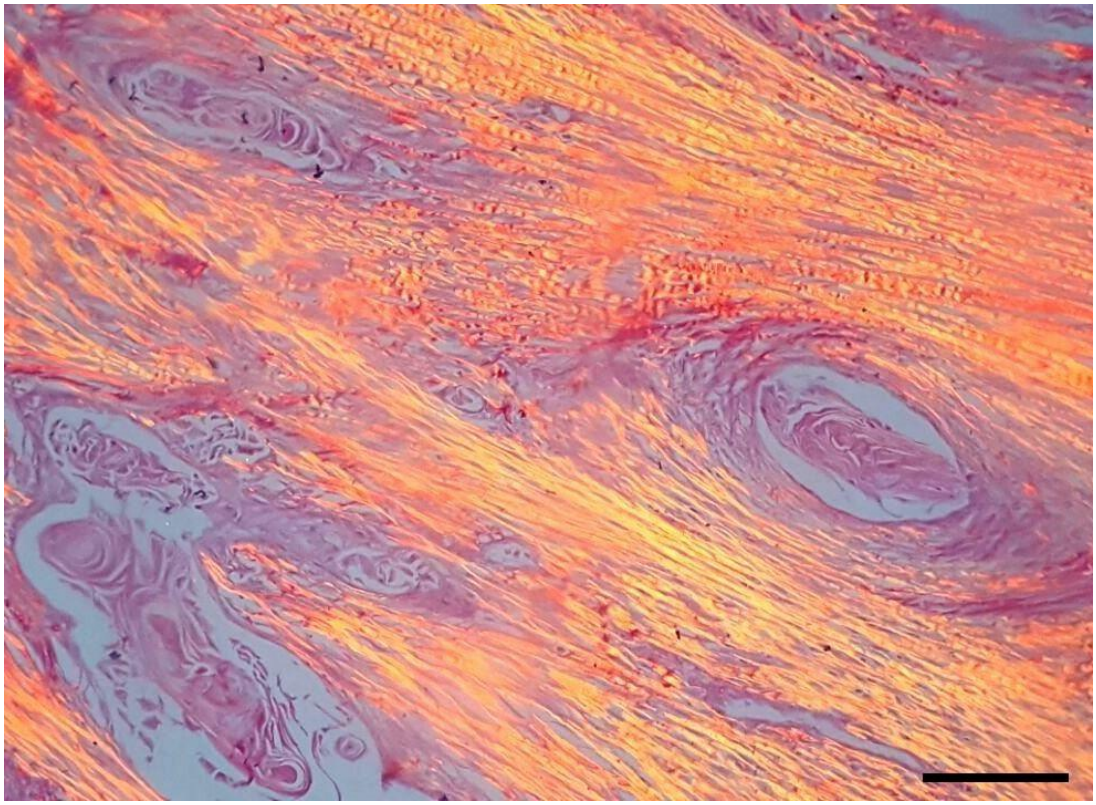

Fig. 1 (e)

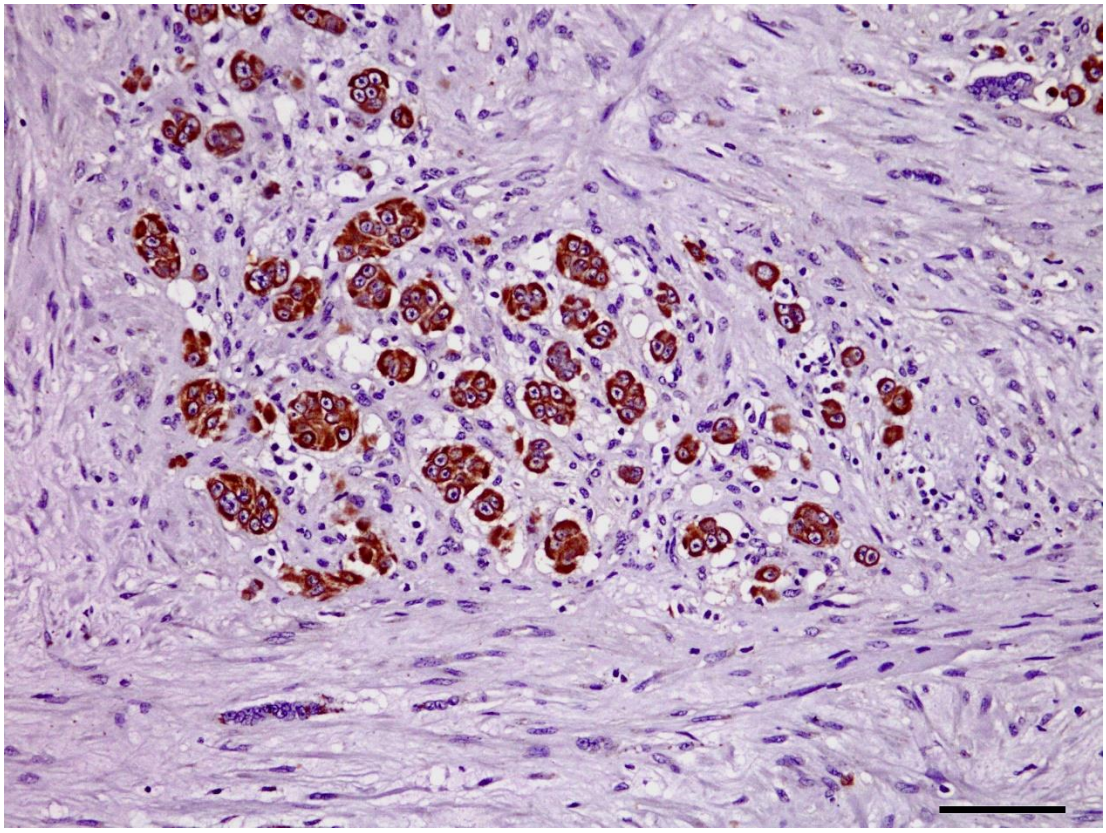

Fig. 1 (f)

4 DISCUSSÃO

Por meio deste estudo foi possível inferir que, em um período de 12 anos, foram recebidos no LPV-UFSM 100 bovinos com CCEs no TAS devido à intoxicação crônica por samambaia, evidenciando que essa forma de câncer não é rara em bovinos na região de abrangência do LPV-UFSM. Estes animais foram provenientes apenas dos municípios de Jaguari, Nova Esperança do Sul e Santiago, que pertencem à região Central do Rio Grande do Sul. Em todo o período estudado as necropsias foram realizadas de forma padronizada, com metodologia específica que enfatizou a avaliação detalhada de todo o TAS e linfonodos drenantes. A avaliação deste conjunto de casos permitiu a compilação de dois artigos científicos (Artigos 1 e 2).

No Artigo 1, pode-se observar que a maioria destes tumores ocorrem em vacas, adultas, com cerca de 8 anos de idade. Caquexia, atonia ruminal e disfagia foram os sinais clínicos mais comumente observados, semelhante ao já observado por outros autores (SOUTO et al., 2006a; TOKARNIA; DÖBEREINER; CANELLA, 1969; UZAL; PLATTNER; HOSTETTER, 2016). Os sinais clínicos apresentados estão estatisticamente associados com a localização do tumor. Tosse é um sinal clínico associado com tumores na cavidade oral e faringe, regurgitação está associada à CCEs esofágicos e timpanismo aos neoplasmas ruminais. Os CCEs pouco diferenciados apresentaram as maiores taxas de metástases, o que torna o grau de diferenciação histológico o principal fator para predizer o prognóstico desses neoplasmas. Os tumores ocorreram predominantemente como massas endofíticas, o que não tinha sido computado em estudos anteriores realizados pelo grupo de pesquisa.

Os CCEs cutâneos geralmente tem crescimento lento e, embora invasivos, tem baixo potencial metastático para os linfonodos regionais (GOLDSCHMIDT; GOLDSCHMIDT, 2017). Neste estudo, os CCEs alimentares em bovinos se comportaram de maneira mais agressiva, apresentando uma taxa de metástase de 52%, predominantemente nos CCEs pouco diferenciados. Metástases para linfonodos regionais são encontradas com maior frequência em CCEs pouco diferenciados ou em neoplasmas que permanecem um tempo considerável antes de serem diagnosticados ou excisados (GOLDSCHMIDT; GOLDSCHMIDT, 2017). Esses dois fatores podem ter contribuído para a alta frequência de metástases no presente estudo, pois os tumores em bovinos são diagnosticados tardiamente, quando o animal mostra sinais clínicos evidentes devido a grandes massas neoplásicas ou massas (ainda que não muito extensas) que comprometem áreas funcionalmente críticas do TAS, como a entrada do rúmen, por exemplo.

A ausência de amplificação do DNA do PV pelo método de PCR utilizado, a ausência de imunorreatividade nuclear para PV pelo método de IHQ disponível e a ausência de corpúsculos de inclusão intranucleares em papilomas foram juntos sugestivos de que os papilomas do TAS podem não estar associados ao BPV-4 em bovinos da região estudada. Além disso, a presença de lesões intraepiteliais escamosas encontradas ao longo do epitélio do TAS que não parecem passar por um estágio papilomatoso podem demonstrar uma possível ação direta de carcinógenos da samambaia no epitélio, sem a necessidade de envolvimento viral como sugerido por Masuda (2010). Adicionalmente, sabe-se que a persistência da papilomatose alimentar, com progressão para CCEs, é apoiada por imunossupressão por linfopenia causada pela toxicidade da samambaia (CAMPO et al., 1994), porém no Sul do Brasil, linfopenia tem sido pouco observada em animais com esses neoplasmas (MASUDA et al., 2011b). Todos esses fatores suportam a possibilidade de que o BPV-4 pode não ser um dos fatores envolvidos na carcinogênese de CCEs alimentares em bovinos em nossa região, e que os papilomas e CCEs podem ser independentemente induzidos por samambaia. Esta possibilidade também foi considerada em uma revisão de PV (MUNDAY, 2014). Todas essas evidências indicam a necessidade de novas investigações sobre a etiopatogenia desta doença.

No Artigo 2, pode-se constatar que embora possa ser facilmente diagnosticado de forma equivocada como um tecido cicatricial esofágico, os CCEs esofágicos anelares estenosantes de bovinos que pastoreiam em *P. arachnoideum* podem ter uma aparência macroscópica distinta que deve ser incluída no diagnóstico diferencial de estenose esofágica por outras causas. Esses CCEs esofágicos anelares estenosantes apresentaram uma reação desmoplásica fibrosa moderada a acentuada, bem caracterizada pela coloração de Tricrômico de Masson e as fibras de colágeno observadas nesta reação são compatíveis com o colágeno tipo I, caracterizada através da coloração de Picrosirius com luz polarizada; A severidade da desmoplasia nos CCEs esofágicos em humanos é variável (GABBERT et al., 2000) e não foram encontrados casos semelhantes descritos em outras espécies animais. O colágeno de tipo I possivelmente contribuiu para as características estenosantes dos CCEs esofágicos analisados neste estudo.

5 CONCLUSÕES

- Uma associação estatisticamente significativa entre os sinais clínicos e a localização do tumor no TAS foi estabelecida. A tosse é um sinal clínico associado com tumores na região orofaríngea, a regurgitação está relacionada com neoplasmas esofágicos e o timpanismo com CCEs ruminais.

- Há também uma associação significativa entre o grau de diferenciação histológica e a localização do tumor no TAS; os CCEs bem diferenciados são estatisticamente mais frequentes na região orofaríngea, assim como os pouco diferenciados nas regiões esofágicas e ruminais.

- Há uma tendência a associação entre o grau de diferenciação histológica e a presença de metástases. Desta forma, o grau de diferenciação histológica é o principal fator para predizer o prognóstico desses neoplasmas, pois os CCEs pouco diferenciados metastatizam com maior frequência (76,47%) em relação aos moderadamente (52,38%) e bem diferenciados (45,16%).

- A média de idade dos bovinos com CCEs orofaríngeos foi 7,39 anos de idade, com diferença significativa em relação aos bovinos com CCEs esofágicos (8,6 anos).

- Nenhuma associação estatística foi observada entre os outros parâmetros clínico-patológicos analisados (sexo, padrão de crescimento e sítio primário do tumor).

- Estes neoplasmas se apresentam como CCEs, somente no TAS e ocorrem predominantemente como massas endofíticas e em vacas adultas.

- Os tumores são observados predominantemente na região orofaríngea, seguidos pelas regiões ruminal e esofágica.

- Os resultados mostram que não foram observados indícios de papillomavírus nos papilomas do TAS em bovinos da região estudada. Consequentemente, o BPV-4 pode não ser um cofator no desenvolvimento dos CCEs nos bovinos do Sul do Brasil.

- A apresentação anelar estenosante é um tipo distinto de CCE esofágico de bovinos com intoxicação crônica por samambaia.

- As fibras de colágeno tipo I contribuem para as características estenosantes deste tumor esofágico.

- Esta apresentação deste neoplasmas esofágico deve ser considerada no diagnóstico diferencial de estenose esofágica em bovinos.

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